An Investigation of the role of the microbiome in the development of glaucoma

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy in Medicine

University of Newcastle, NSW, Australia

July 2019

This research was supported by an
Australian Government Research Training Program (RTP) Scholarship

Statement of Originality

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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Acknowledgement of Authorship

I hereby certify that the work embodied in this thesis contains published papers and scholarly work of which I am a joint author. I have included as part of the thesis a written declaration endorsed in writing by my supervisor, attesting to my contribution to the joint publications and scholarly work.

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Declaration of Joint Authorship

By signing below, I confirm that Zachary E. McPherson was a co-first author to the publication entitled 'Host-microbe interactions: The aryl hydrocarbon receptor and the central nervous system', displayed in Chapter 2, and his contribution consisted of: developing the core ideas of the publication, performing the literature review which served as the basis of the publication, drafting the manuscript with co-first author Dr Hae Ung Lee, and editing the manuscript in collaboration with the co-authors.

By signing below, I confirm that Zachary E. McPherson was the primary author to the research that is displayed in Chapter 3 of this thesis entitled 'Adults with Glaucoma are More Likely to also Have IBS' and his contribution consisted of: developing the core hypothesis, developing of the methods and collaboration in the required ethics submission, analysing the dataset, and drafting the chapter. Collaborators participated in data collection and advised on data analysis particularly with regards to diagnosis of IBS made by survey results.

By signing below, I confirm that Zachary E. McPherson was the first author to the manuscript entitled 'Irritable bowel syndrome and risk of glaucoma: an analysis of two independent population-based cohort studies', displayed in Chapter 4, and his contribution consisted of: developing the core hypothesis, developing the methodology, acquisition of the 1958 United

Kingdom Birth Cohort (UKBC) data, analysing the UKBC dataset, collaboration and advice on analysis in the Danish National Patient Registry (DNPR), analysis of residual confounding, and drafting of the manuscript.

By signing below, I confirm that Zachary E. McPherson was a contributing author to the publication entitled 'Prospective study of oral health and risk of primary open-angle glaucoma in men: data from the Health Professionals Follow-up Study', displayed in Chapter 5, and his contribution consisted of: analysis of results and participation in developing the manuscript.

By signing below, I confirm that Zachary E. McPherson was the primary author to the research that is displayed in Chapter 6 of this thesis entitled 'The Microbiome is Protective in Optic Nerve Crush in Mice' and his contribution consisted of: developing the core hypothesis, developing of the methods, drafting the required ethics submission, performing the optic nerve crush procedures, sample collection, performing the wetlab research, analysing data, and writing the chapter. Collaborators participated in animal husbandry, some sample collection, advice on technical methods, and advice on data interpretation.

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Acknowledgements

To my supervisors, Professor Nick Talley, Professor Sven Pettersson and Associate Professor Mark McEvoy, without your guidance, support and insight, this project would never have been possible. Mark, who would have thought that a cup of tea five years ago would have led to all of this. Your encouragement of my work and me as a researcher is so valued, and your willingness to go along with my unusual ideas is the reason we have gotten this far. Thank you for all that you have done to support me in this project. To Sven, thank you for you accepting me into your laboratory. Your guidance with my animal research has been invaluable and led to a project that I am very proud of. Similarly, to Nick thank you for bringing a critical eye to the oversight of my work. Despite your workload you have always been willing to put effort into my work even when my project has drifted quite some distance from the gut. To each of you, thank you for your help whipping this project into shape. Thank you for helping me troubleshoot the issues that popped up along the way. And finally, thank you for walking through this process with me.

To my lab mates and colleagues, one of the best things about the way research is done is the teams that work together to get the job done. You have all taught me new techniques and processes, but you've also taught me so much more about the world around me. You've given me greater perspective and I'm glad that we have become friends along the way.

To the clinicians and researchers who have supported me in my career until now, particularly Dr Ashish Agar, Dr Minas Coroneo, Dr Ian Francis, and Dr Andrew White. Your advice and continued support has been indispensable. Without your help I would not have been able to produce a project of this calibre.

To Jennie Thomas and the Barker family, your financial support has enabled me to pursue my research in Singapore, and attend conferences and courses in the UK, the USA and Asia. I am thankful that, because of your support, my mind remained free of financial stress and able to pursue this project with all of my being. I hope that through my work, your generous commitment to scientific advancements leads to benefits for the world.

To Pastor Tan Seow How and Pastor Cecilia Chan, thank you for bringing Imogen and I into your family. We thought Singapore was only ever meant to be a chapter in our lives but now more than anywhere it's our home. This project may have brought us to you, but you've helped me get so much more out of it and myself than I could have imagined. Thank you for directing my focus and keeping me aligned with what truly matters.

To my friends, Scott and Sam, you've answered phone calls at weird times of day to talk about anything and everything unrelated to this project. You've kept me sane and grounded and you are two of the greatest men a guy could be friends with.

To my family, your love and support has been constant. I *really* wouldn't have been able to do this without you.

To Malachi and your unborn brother, being a father has certainly slowed progress on this project, but you two are my greatest achievement, which makes this project number three on the list after being your Dad and convincing your Mum to marry me.

Finally, and most importantly, to Imogen, my bride, it's finished, we did it!

Publications and Presentations

At the time of presentation, two articles have been published from the contents of this thesis with one further article under consideration. Additionally, the research presented in this thesis has been presented at a number of international conferences in the form of oral presentations and poster presentations.

Manuscripts published:

Lee HU*, **McPherson ZE***, Tan B, Korecka A, Pettersson S. Host-microbiome interactions: the aryl hydrocarbon receptor and the central nervous system. J Mol Med 2017:95(1);29-39
**co-first authors*

Pasquale LR, Hyman L, Wiggs JL, Rosner BA, Joshipura K, McEvoy M, **McPherson ZE**, Danias J, Kang JH. Prospective Study of Oral Health and Risk of Primary Open-Angle Glaucoma in Men: Data from the Health Professionals Follow-up Study. Ophthalmology 2016:123(11);2318-2327

Under Consideration:

McPherson ZE, Sørensen HT, Horváth-Puhó E, Agar A, Coroneo MT, White A, Francis IC, Pasquale LR, Kang JH, Pettersson S, Talley NJ, McEvoy M. Irritable bowel syndrome and risk of glaucoma: an analysis of two independent population-based cohort studies. Submission to Gut (under consideration)

Oral Presentations:

McPherson ZE (Presenter), Horvath-Puho E, Toft Sørensen H, Nørgaard M, McElduff P, McElduff S, Agar A, Coroneo M, White A, Wang JJ, Francis IC, Pasquale LR, Kang JH, Craig J, Kelman J, Walker MM, Talley NJ, Pettersson S, McEvoy M. Irritable Bowel Syndrome is a novel risk factor for glaucoma; Analysis of two European population-based cohort studies. Royal Australia and New Zealand College of Ophthalmology (RANZCO) Annual Scientific Congress. Melbourne, Australia. November 2016

Poster Presentations:

McPherson ZE, Talley NJ, Walker MM, McElduff P, Attia J, Kelly B, Agar A, Coroneo MT, Pettersson S, Sørensen HT, White A, McEvoy M. A Novel Predictive Association between Irritable Bowel Syndrome and Glaucomatous Optic Neuropathy. Digestive Diseases Week. Washington DC, USA. May 2015

McPherson ZE, McEvoy M, Lee HU, Talley N, Agar A, Coroneo M, Pettersson S. Connecting the microbiome to tissue repair mechanisms in a mouse model of Glaucoma. Bridging Biomedical Worlds: Frontiers in Human Microbiota Symbiotic Interactions. Hong Kong, Hong Kong. May 2016

McPherson ZE, McEvoy M, Lee HU, Talley N, Agar A, Coroneo M, Pettersson S. Alteration of the microbiome effects neuroprotective mechanisms in an animal model of Glaucoma. Falk Symposium 207 'Gut Microbiome and Mucosal or Systemic Dysfunction: Mechanisms, Clinical Manifestations and Interventions'. Brisbane, Australia. May 2017

Additional manuscripts published in peer-review journals during candidature:

Ewe SY, Abell RG, Oakley CL, Lim CH, Allen PL, **McPherson ZE**, Rao A, Davies PE, Vote BJ. A Comparative Cohort Study of Visual Outcomes in Femtosecond Laser-Assisted versus Phacoemulsification Cataract Surgery. Ophthalmology. 2016:123;178-82

Pattamatta U, **McPherson ZE**, White A. A mouse retinal explant model for use in studying neuroprotection in glaucoma. Exp Eye Res. 2016:151;38-44

Merani R, **McPherson ZE**, Luckie AP, Gilhotra JS, Runciman J, Durkin S, Muecke J, Donaldson M, Aralar A, Rao A, Davies PE. Aqueous Chlorhexidine for Intravitreal Injection Antisepsis: A Case Series and Review of the Literature. Ophthalmology. 2016:123;2588-2594

Jain NS, Liu Y, Wang SB, George A, Govendir M, **McPherson ZE**, Agar A, Francis IC. Teaching Hospital Cataract Surgical Outcomes in Adelaide, Australia. Clin Experiment Ophthalmol. 2016:44;648

Kelman JC, **McPherson ZE**, Sim BW. Projectile fly larvae: A potentially under-reported cause of ocular foreign body sensation and inflammation in Australia. Aust Fam Physician 2017:46;129-130

Spencer SKR, Shulruf B, **McPherson ZE**, Zhang H, Lee MB, Francis IC, Bank A, Coroneo MT, Agar A. Factors Affecting Adherence to Topical Glaucoma Therapy: A Quantitative and Qualitative Pilot Study Analysis in Sydney, Australia. Ophthalmology Glauc 2019:2(2);86-93

Skill Development Arising from this Thesis

Beyond the research outlined in this thesis and the publications and presentations previously outlined, my PhD experience has developed me as a researcher in several significant skill areas. The experiences I have gained, outlined below, have given me a significant insight into the expectations and requirements of a researcher and have honed my research abilities for my career ahead.

Written communication skills:

- During my candidature I have produced several articles in peer-reviewed journals.
- I have been involved in the writing of ethics proposals for both human and animal research ethics boards in three separate institutions across three different nations.
- I have written proposals for and successfully obtained three competitive grants (two Jennie Thomas Travel Scholarships and the Barker Family scholarship). In addition to this I have been involved in the drafting of 2 additional major grants for projects led by other members of my lab in Singapore.

Oral communication skills:

- I have presented one oral presentation and three posters at international conferences over the time of my candidature. Each of these conferences allowed me to engage with and debate my research with leading researchers in an array of fields relevant to my research.
- Participation within a research group (Microbiota Host Interactions Laboratory, NTU Singapore) required me to present my findings at least 4 times per year allowing me to develop my presentation skills whilst also developing my research.
- The laboratory consisted of a number of researchers, technical staff and often a student on a research attachment. This mix of individuals taught me how to communicate with people from a wide variety of cultural backgrounds. Furthermore, this also gave me the opportunity to teach a number of the skills I had developed to other members of the lab to help assist with their own work.

Teamwork and collaboration:

- Through this research I was able to successfully negotiate collaborations with researchers in the United Kingdom, Denmark, Singapore, the United States and also in two other institutions in Australia.
- International and multi-institutional collaborative work has developed my skills in communication, leadership and time management. Indeed, reaching consensus on the presentation of results is an important task when performing collaborative research and the findings I have presented in this thesis are the result of this skill development.
- The collaboration I set up with Nanyang Technological Institute has led to further discussions between a number of research groups within the two institutions with promise for future research projects beyond the work I have done.

Technical skill acquisition:

- Learning how to use statistical software: STATA and Prism
- Learning how to design and analyze prospective cohort study data from datasets based on different study designs
- Learning how to conduct case control studies
- Learning how to use of Directed Acyclic Graphs for the identification of confounding factors and indirect causal pathways.
- Learning how to handle Germ Free mice, and developing my skills in handling of Specific Pathogen Free mice.
- Learning how to perform an Optic Nerve Crush in mice
- Learning how to perform an intravitreal injection in mice
- Developing of skills in quantitative PCR and Immunohistochemistry wet lab skills
- Learning how to perform ELISA and Western Blot analysis of protein
- Learning how to perform Chromatin Immunoprecipitation
- Learning how to perform in situ Hybridization
- Learning how to troubleshoot when protocols don’t perform as expected.

- Learning how to problem solve when replications of experiments do not conform with previously collected data
- Learning to develop and modify hypotheses based on the acquisition of new data
- Development of my ability to read and understand the literature and gain an understanding of how my own research fits into the current literature

Table of Contents

List of Tables

List of Figures

List of Commonly Used Abbreviations

Aβ ..... Amyloid Beta
AAV ..... Adenovirus-Associated Vectors
AD ..... Alzheimer's Disease
AHR ..... Aryl Hydrocarbon Receptor
ALS ..... Amyotrophic Lateral Sclerosis
ANOVA ... Analysis of Variance
ANZRAG . Australia and New Zealand Registry of Advanced Glaucoma
ATC ..... Anatomical Therapeutic Classification
AUC ..... Area Under the Curve
BBB ..... Blood Brain Barrier
BDNF ..... Brain Derived Neurotrophic Factor
BP ..... Blood Pressure
BSA ..... Bovine Serum Albumin
CCT ..... Central Corneal Thickness
cDNA ..... complementary DNA
CFU ..... Colony Forming Units
CI ..... Confidence Interval
CNS ..... Central Nervous System
CON ..... GF mouse conventionalised with faecal microbiome from SPF mouse
COPD ..... Chronic Obstructive Pulmonary Disease
CRP ..... C reactive protein
CSF ..... Cerebrospinal Fluid
DNA ..... Deoxyribonucleic Acid
DNPR ..... Danish National Patient Registry
ELISA ..... Enzyme-Linked Immunosorbent Assay
FISH ..... Fluorescent in situ Hybridisation
FMT ..... Faecal Matter/microbiome Transplant
FODMAP Fermentable Oligo-, Di-, Mono-saccharides And Polyols
GF ..... Germ Free
GIT ..... Gastrointestinal Tract
GWAS ..... Genome Wide Association Study
HCS ..... Hunter Community Study
HPA axis . Hypothalamic-Pituitary Adrenal axis
HPFS ..... Health Professionals Follow-up Study
IBS ..... Irritable Bowel Syndrome
IBS-C ..... Constipation prominent IBS
IBS-D ..... Diarrhea prominent IBS
IBS-M ..... IBS with both constipation and diarrhea (Mixed)
ICD ..... International Classification of Diseases
Ig ..... Immunoglobulin
IL ..... Interleukin (numbered)
IOP ..... Intraocular Pressure
LPS ..... Lipopolysaccharide
MCAO .... Middle Cerebral Artery Occlusion
mRNA ..... Messenger-RNA

MS ..... Multiple Sclerosis
MSA ..... Multiple Systems Atrophy
MVRR ..... Multivariable Relative Risks
NF-κB ..... Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGF ..... Nerve Growth Factor
NT-3 ..... Neurotrophin-3
NT-4 ..... Neurotrophin-4
NTG ..... Normal Tension Glaucoma
OSA ..... Obstructive Sleep Apnoea
ONC ..... Optic Nerve Crush
OR ..... Odds Ratio
p75NTR .. Low-affinity nerve growth factor receptor
PD ..... Parkinson's Disease
PI-IBS ..... Post Infectious IBS
POAG ..... Primary Open Angle Glaucoma
qPCR ..... Quantitative Polymerase Chain Reaction
RBPMS ... RNA Binding Protein with Multiple Splicing
RCT ..... Randomised Control Trial
RGC ..... Retinal Ganglion Cell
RNA ..... Ribonucleic acid
rRNA ..... Ribosomal RNA
SCFA ..... Short Chain Fatty Acid
SEM ..... Standard Error of the Mean
SNP ..... Single Nucleotide Polymorphism
SPF ..... Specific Pathogen Free
TLR4 ..... Toll Like Receptor 4
TNFα ..... Tissue Necrosis Factor Alpha
Trk ..... Tyrosine Kinase receptor (sequentially lettered)
UKBC ..... United Kingdom Birth Cohort 1958
VF ..... Visual Field

A Note on Style

This thesis follows standard nomenclature formatting as suggested by the relevant guidelines[1, 2]. Briefly, genes (including reference to mRNA and cDNA) will be italicised and fully capitalised when referring to human genes; and italicised with the first letter capitalised when referring to rodent genes. Proteins will be fully capitalised (without italics) for both rodent and human proteins. Regarding bacterial species, the first instance will spell out the relevant genus, and subsequently the genus will be abbreviated.

Thesis Abstract

Glaucoma is a neurodegenerative illness of the optic nerve with only one treatment pathway due to the lack of clear modifiable factors. Amongst its pathophysiological mechanisms, neurotrophic factor deprivation [particularly of Brain Derived Neurotrophic Factor (BDNF)] and inflammation are mechanisms that may present therapeutic opportunity. Safely modulating the endogenous neurotrophic mechanisms or immune pathways may be suitable therapeutic pathways in future.

The microbiome is now clearly understood to be crucial to the development of the host. In animal research the links between microbiome status and host physiology are becoming increasingly clear. It is now known that the microbiome plays an important role in the central nervous system with the ability to regulate neurotrophins and the neuro-immune system (amongst other mechanisms). As these mechanisms are important in glaucoma pathophysiology, the central hypothesis of this study is that the microbiome contributes to glaucoma.

This thesis presents a series of studies that begin the process of linking glaucoma to the microbiome. The research presented in this thesis falls broadly into two categories: human observational epidemiology and experimental animal research.

Epidemiological Research

As human microbiome research represents a data analysis problem, illnesses that are clearly related to abnormal microbiome should be useful markers of altered microbiome in epidemiological research. Irritable Bowel Syndrome (IBS) and dental illness are both very strongly correlated to abnormal microbiome in the gastrointestinal tract and the oral cavity, respectively.

The first study (Chapter 3) aimed to quantify the prevalence of IBS in an Australian cohort of glaucoma sufferers as compared to the general Australian population. Participants from the Australia and New Zealand Registry of Advanced Glaucoma (n=1021) and a population representative cohort, the Hunter Community Study (n=2251), returned a mailed survey with the ROME-III criteria for the diagnosis of IBS. The participants with glaucoma were also significantly more likely to have ROME-III defined IBS [Odds Ratio (OR) 1.93, 95% Confidence Interval (CI) 1.52-2.44].

The second study (Chapter 4) aimed to identify and quantify an increased incidence of glaucoma in people with IBS in two large population based European cohorts. In the 1958 UK Birth Cohort, participants (n=9091) were surveyed regularly regarding their health. Amongst people who had IBS at 42 who continued to report their illness at age 50, the adjusted odds ratio of developing glaucoma in this period was 5.84 (95% CI 2.26-15.13). In the Danish National Patient Register (n=62,541 with IBS, 625,410 general population controls), people with IBS had a hazard ratio (HR) of 1.35 for developing physician-diagnosed glaucoma (95%CI 1.15-1.59), a HR of 1.34 for undergoing surgery for glaucoma (95%CI 1.04-1.74), and a HR of 1.19 for initiating use of glaucoma medication (95%CI 1.02-1.40). These effects were similar in lagged analyses, and when Cholelithiasis was used as a negative control.

A third investigation (Chapter 5) was undertaken to identify and quantify the size of an association between dental illness (periodontitis and incidental tooth loss) and the incidence of glaucoma. In the Health Professionals Follow-up Study participants (40,536 men) followed biennially from 1986 to 2012, the number of natural teeth, teeth lost, periodontal disease and root canal treatments was followed with assessment of glaucoma incidence as its outcome. Incident tooth loss was associated with receiving a glaucoma diagnosis in the following two years (Risk Ratio (RR) 1.45, 95% CI 1.06-1.97), especially if the tooth loss was in the context of periodontal disease (RR: 1.85, 95% CI 1.07-3.18). The total number of teeth, periodontal disease (alone) and root canal treatment were not related to glaucoma incidence.

Animal Research

Although there are several microbiome manipulation models, Germ Free (GF) mice [when compared to Specific Pathogen Free (SPF) mice and Conventionalized GF (CON) mice] are the best model for assessing the role of the normal microbiome. Similarly, the Optic Nerve Crush (ONC), is a reproducible optic nerve injury model of glaucoma, that allows researchers to investigate the pressure independent mechanisms at work in retinal ganglion cell (RGC) neurodegeneration in mice.

In the study presented in Chapter 6, GF, SPF and CON mice were subjected to ONC, and allowed to survive until their retinae were harvested for analysis (up to 3 days for protein analysis, 1 week for qPCR and 5 weeks for cell survival analysis). Immunohistochemistry was used to examine the cell survival, and qPCR and ELISA protein analysis were used to quantify the BDNF levels in the retina, at various time points after the ONC. A further cohort of GF

mice were treated with live or heat-killed *Lactobacillus* probiotic, and its effects on cell survival after ONC were quantified. Finally, a cohort of GF and SPF mice that also received an injection of BDNF protein at the time of ONC and its effects were compared to mice who received a placebo injection.

GF mice had significantly worse RGC survival at 7 days (RGC survival of 40.5% compared to 50.4% and 48.4% for SPF and CON mice, respectively, $p<0.05$) and at 35 days (RGC survival of 11.8% compared to 18.1% and 18.8% for SPF and CON mice, respectively, $p<0.05$) after initiation of ONC.

Probiotic supplementation for GF mice with *Lactobacillus plantarum* PS128 was able to increase cell survival after ONC. At day 35 after ONC, cell survival in live probiotic treated mice was 16.2% compared to GF mice with 11.8% survival ($p=0.04$). When the probiotic was heat-killed the RGC cell survival was insignificantly elevated compared to GF mice (12.5%).

At day 3 after ONC, it was shown that SPF mice had 34.6% greater expression of BDNF protein as compared to GF mice ($p<0.001$), however protein levels at baseline and mRNA levels at all timepoints were no different. To evaluate if the differentially expressed BDNF may be responsible for differential cell survival between SPF and GF mice, a single intraocular injection of recombinant BDNF was administered at the time of ONC. The BDNF injection was protective in both SPF and GF mice, and importantly it normalised the cell survival rates between SPF and GF mice after ONC [at day 35, cell survival was 22.4% and 19.9%, respectively ($p=0.61$)].

Conclusions and Discussion

These epidemiological studies together show that IBS and perhaps dental illness (both illnesses associated with abnormal microbiome), are risk factors for glaucoma. Although the microbiome is not certainly the mechanism linking these entities, as there is limited plausible overlap in the physiology of these illnesses aside from the microbiome these findings are evidence towards the hypothesis that the microbiome is relevant to glaucoma's pathology. The animal research presented demonstrated conclusively that the absence of microbiome leads to poorer outcomes after ONC, an optic nerve injury model of glaucoma. These findings also suggest that microbiome dependant effects on retinal BDNF levels after ONC may be the reasons for this protective effect. Although these findings require further investigation, they also support the hypothesis that the microbiome is involved in neuroprotective mechanisms

in glaucoma. In summary, this thesis provides epidemiological evidence that the microbiome may be clinically relevant to glaucoma incidence; also, animal research suggests that a BDNF mediated mechanism could underly this effect.

Thesis Structure

This thesis is organised into four main sections:

Section 1: Chapters 1 and 2 provide the background to the fields of research drawn on in this research.

Section 2: Chapters 3 to 5 investigate the role of microbiome related illnesses as risk factors for glaucoma.

Section 3: Chapter 6 investigates the role of the microbiome in the neuroprotection of retinal ganglion cells after optic nerve crush in mice

Section 4: Chapters 7 and 8 summarises and discusses the findings of this thesis with a view to how this research impacts the field at present and how this may be developed in the future.

Section 1 – Background

Chapter 1 – Introduction and Literature Review

1.1 Glaucoma

Glaucoma is a neurodegenerative disorder characterised by damage of the optic nerve at the optic nerve head. Its pathognomonic signs are the alteration of the appearance of the optic nerve head and associated reduction of visual field (VF) sensitivity (Figure 1.1). It has often been referred to as 'the sneak thief of sight, as VF loss begins in the peripheral vision with many patients unaware of their disease until a significant proportion of their vision is lost[3]. The primary pathology is the apoptosis of retinal ganglion cells (RGCs), the output neurons of the retina, responsible for passing visual information to the brain.

Glaucoma can be defined into different clinical diagnoses. The most common types of glaucoma in Australia and around the world[4-6] are:

- Primary Open Angle Glaucoma (POAG) – defined by glaucomatous damage of the optic nerve in the presence of elevated intraocular pressure (IOP), and clinically normal anterior chamber drainage systems.
- Normal Tension Glaucoma (NTG) – defined by glaucomatous damage of the optic nerve in the absence of elevated IOP
- Angle Closure Glaucoma – defined by glaucomatous damage of the optic nerve due to the very high IOP caused by the closure of the anterior chamber drainage systems
- Secondary Open Angle Glaucoma – defined by glaucomatous damage of the optic nerve in the presence of other pathological mechanisms that may raise the patients IOP by compromising the anterior chamber drainage systems such as pseudoexfoliation syndrome (where abnormal proteins are deposited blocking the drainage system) or rubeosis (where abnormal vessels form, blocking the drainage system)

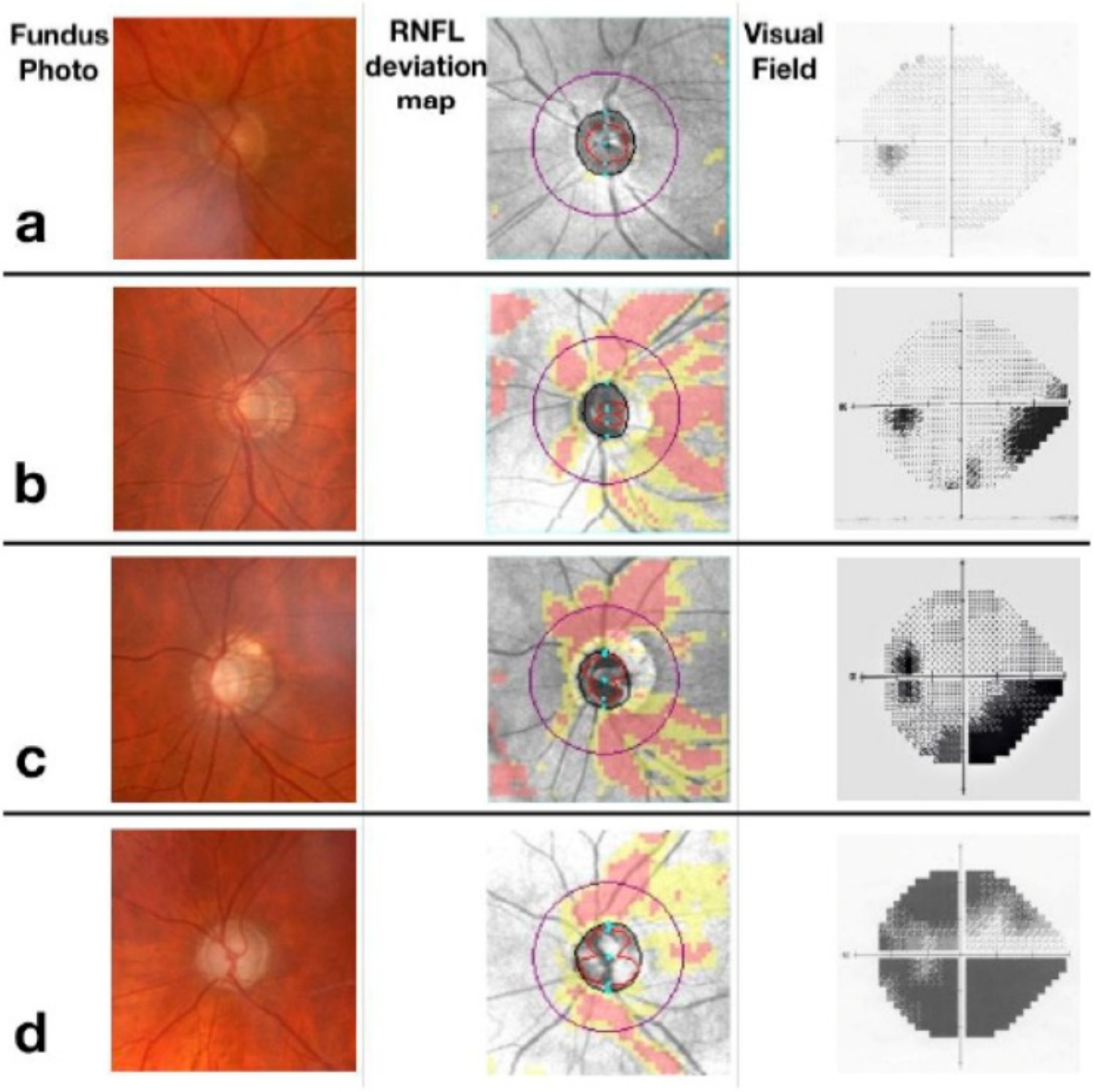


Figure 1.1: The characteristic progression of glaucoma
Stages of glaucoma increasing in severity from (a) normal eye through to (d) severe glaucoma. Note the progression in excavation of the optic nerve head in the fundus photography, the increasing Retinal Nerve Fibre Layer (RNFL) loss, and the progression of peripheral vision loss on visual field analysis. Figure reproduced according to the CC-BY-NC 4.0 license from Sacca *et al*. Nutrients 2019:11;239[7].

This thesis primarily focuses on POAG and NTG. Due to the similar pathology, there is much conjecture regarding the distinction of NTG from POAG[8]. Some suggest NTG is caused by elevated sensitivity to IOP, whilst other ophthalmic scientists cite the difference in NTG's risk factors (i.e. specific genetic markers and hypoxic pathologies) as evidence of different pathological pathways occurring in these patients[8]. Regardless, treatment for NTG involves the same options as POAG. As clinically and scientifically there is no convincing data to suggest that they are distinct entities, for the purposes of this thesis, they will be considered on a continuum of the same illness, and the term 'glaucoma' will be used to refer broadly to both these types of glaucoma, with other secondary or acute forms of glaucoma noted when discussed.

The burden of glaucoma is best appreciated by the fact that it remains the leading cause of irreversible blindness worldwide[9-11]. A 2017 meta-analysis shows that it is the 4th largest cause of moderate to severe vision impairment (vision worse than 6/18, up to 3/60 in the better eye) behind the treatable conditions of refractive errors, cataract and age-related macular degeneration[11]. It is the third most common cause of blindness (vision worse than 3/60 in the better eye) behind cataract and refractive error[11]. Glaucoma is responsible for moderate to severe vision impairment or blindness of almost 7 million people world wide[11]. The 20-year risk of unilateral blindness due to glaucoma has been quantified at 27-40.5% and bilateral blindness at 9-22%[12, 13]. Treatment options currently focus on intraocular pressure[14]. Despite being a neurodegenerative disease, neuroprotective therapies have not yet demonstrated any clinical benefit[15].

1.1.1 Risk Factors for Glaucoma

Age

Glaucoma, like most neurodegenerative disorders, is primarily an illness of aging. A 2004 meta-analysis found the prevalence of glaucoma in the population of the United States of America (USA) was between age 40 and 49 to be 0.68% which rapidly rose with age until in the population over 80 years old it was found to be 7.74%[16]. A 2014 meta-analysis showed that each decade of age increase corresponded to an Odds Ratio (OR) of 1.73 for glaucoma prevalence[17].

Family History, Ethnicity and Genetics

Family history has been recognised as a risk factor for glaucoma since the mid 19th century[18]. With modern epidemiological research, family history has been shown to be a significant risk factor for incident glaucoma in many studies[19-22]. In one study, the 1st degree relatives of a glaucoma patient were 9.2 times more likely than controls to develop glaucoma[21]. In a relatively sizeable Australian cohort, 59.6% of patients with glaucoma had a relative with the illness[22].

Ethnicity has also been associated with glaucoma. A 2014 meta-analysis found that African heritage was significantly associated with 2.8 increased odds of having glaucoma, with Asian and Hispanic heritage demonstrating an insignificantly elevated rate of glaucoma compared to those of European decent[17]. In the same study, Asian heritage was significantly associated with Primary Angle Closure Glaucoma but not POAG[17]. Even so, open-angle glaucoma appears to take on a different phenotype in Asians with a higher prevalence of NTG in these populations[23] (up to 92% of all glaucoma in Japan[24]) and also a higher prevalence of narrow-angle glaucoma[23]. Interestingly although people of African descent appear to have the highest rates of glaucoma, the association with age appears to have its steepest relationship in Caucasian populations[25].

Mendelian familial glaucoma has been described for well over a century[26]. Pedigree studies have identified mutations in the following genes as causative for glaucoma: *MYOC*[27, 28], *OPTN*[29, 30], *TBK1*[31], and *WDR36*[32]. Genome-wide association studies (GWAS) have allowed for understanding the genetics behind sporadic diseases. The first GWAS for glaucoma genetics was reported in 2009[33], although none of the reported loci achieved genome-wide significance ($p < 5 \times 10^{-8}$). Since then, GWAS studies have identified numerous single nucleotide polymorphisms (SNPs) associated with glaucoma[34-48]. Functional analysis of these genes has been suggested as a way to identify the pathological relevance of the GWAS findings. The genes identified are associated with cytokine signalling (*CDKN2B*[49], *TGFBR3*[50], *FNDC3B*[51], *MAP3K1*[52], *MEIS2*[53]), lipid metabolism (*CAV1/CAV2*[54], *ABCA1*[55], *ARHGEF12*[46], *ELOVL5*[56]), fucose and mannose metabolism (*GMDS*[57], *PMM2*[57]), membrane biology (*CAV1/CAV2*[58]), cell division (*CDKN2B*[59], *TMCO1*[60], *GAS7*[61], *MEIS2*[62]), extracellular matrix maintenance (*AFAP1*[63], ocular development (*SIX1/SIX6*[64], *FOXC1*[65], *MAP3K1*[66], *LMX1B*[67], *HMGA2*[68]), oxidative stress defence (*TXNRD2*[69]), and autophagy (*OPTN*[70], *TBK1*[70], *TXNRD2*[71]).

Gender

The association between glaucoma and gender is unclear. A 2006 meta-analysis found that glaucoma was 1.37 times more common in men than women [with 95% Confidence Interval (CI) of 1.22-1.53][25]. Since the publication of this meta-analysis a number of studies have been published that have shown similar results[72, 73], or no significant association[74-76] between gender and glaucoma. Due to increased life expectancy there are more women worldwide with glaucoma than men[77].

Intra Ocular Pressure

Perhaps the most well-known of glaucoma's risk factors, the only clinically significant modifiable risk factor for glaucoma is elevated IOP. Ocular hypertension is defined by IOP greater than 21mmHg, but it is clear that the relationship between intraocular pressure and glaucoma risk is not confined to this binary status. The Ocular Hypertension Treatment Study demonstrated that each mmHg increase in IOP, increased the hazard of developing glaucoma [Hazard Ratio (HR) 1.10, 95%CI 1.05-1.17][78]. The Early Manifest Glaucoma Trial found that mean IOP at follow-up was related to progression with each mmHg of IOP increasing the risk of progression by 13% (HR 1.13, 95%CI 1.07-1.19)[79].

The Collaborative Normal-Tension Glaucoma Study followed 140 patients diagnosed with NTG (average IOP of 16mmHg in untreated patients). Patients randomized to IOP lowering treatment (with a target of 30% IOP reduction) had less than half the rate of progression compared to untreated patients demonstrating a role for pressure in glaucoma with apparently 'normal' IOPs[80, 81]. This study and others similar have been used to argue that POAG and NTG are the same illness.

Anatomy of the Eye

Myopia[82-88] and long axial length[82, 88, 89] are associated with glaucoma. The Singapore Epidemiology of Eye Diseases study showed that IOP and refractive myopia had synergistic effects in their association with glaucoma[90].

The central corneal thickness (CCT) has been suggested as an important ocular measurement that may predict glaucoma[78, 91]. A recent meta-analysis of cross sectional studies demonstrated that people with glaucoma have a significantly lower CCT[92]. A related

biometric measure of the cornea is its hysteresis, which positively correlates with thickness[93]. In two retrospective studies by the same group, a low corneal hysteresis was shown to play a larger role than corneal thickness in predicting glaucoma progression[93, 94].

The morphology laminar cribrosa morphology has been investigated significantly to determine if there is any association between it and glaucoma. The lamina cribrosa is the site of RGC axon damage[95]. Glaucoma is associated with a lower laminar cribrosa thickness[96]. It has also been shown that the radius of the curvature of the lamina cribrosa significantly predicted a quicker rate of glaucomatous progression[97].

Given the role of the optic disc in glaucomatous pathology, it had been theorized that a larger optic disc size might predispose a patient to glaucoma however neither the Ocular Hypertension Treatment study[98] nor a longitudinal study of 763 eyes[99] found associations between disc size and glaucoma.

Corticosteroids

Corticosteroid use corresponds with an elevation in the IOP of a significant proportion of the population, termed 'steroid response'. Indeed, steroids may cause glaucoma through their effects on IOP[100]. A number of animal models of glaucoma have utilised the IOP effects of steroids including in rabbits[101, 102], cows[103, 104] and sheep[105, 106], and are fairly predictable in these species. Amongst the general population approximately 18-36% of the population are steroid responders, the proportion amongst people with glaucoma is substantially higher[100]. There has been some work linking steroid response to genetic markers[107], however the biology behind steroid response, and the reasons why some people ‘respond’ and others don’t is not fully understood[108].

Diabetes Mellitus

The role of diabetes in glaucoma has been hotly debated. Three relatively well-performed meta-analyses have been performed. The studies performed in 2004[109], 2014[110] and 2015[111], which included, 12, 13 and 47 studies respectively each found similar effect sizes of OR between 1.35-1.5 for the association between diabetes and glaucoma. Even so, there is significant bias noted by these meta-analyses[111].

As diabetes guidelines mandate regular ophthalmological screening for retinopathy, any link between diabetes and glaucoma is severely confounded by surveillance bias[112]. A 2016 study showed that diabetes was a risk factor for glaucoma, but fasting glucose (a measure of diabetes severity) was not associated with glaucoma[113]. Interestingly, a study assessing the population-based registries in Denmark demonstrated that diabetes is only a risk factor in glaucoma in the younger population with no significant risk conferred over the age of 80[114].

Smoking

The generally harmful effects of smoking are well known. Despite this, the evidence for an association between smoking and glaucoma has been surprisingly weak. In fact, in a 2017 systematic review that included 17 studies, only six found any significant harmful effect between smoking and glaucoma[115]. Surprisingly, two of the studies found a significant 'protective' effect for smoking in glaucoma[115]. Of the largest studies included; a study of 71,819 people found a trend (p=0.06) for the inverse relationship between pack-years and glaucoma[116]; a study of 32,570 of African American women found that the only significant risk for glaucoma was in participants under 50 years old with greater than 20 pack year history of smoking[117]; and a study of 6,142 people was one of the studies that demonstrated a significant 'protective' relationship between current smoking and glaucoma prevelence[118]. Another meta-analysis of six papers found no relationship between smoking and glaucoma[119]. Since the publication of these reviews, another prospective cohort study of 16,797 participants found that smoking is a statistically significant risk factor for glaucoma, and importantly a dose-response of pack-years was also shown to be significant[120]. In light of these collective findings, the role of smoking and glaucoma remains controversial.

Alcohol

The relationship between alcohol and glaucoma has been looked at in many large studies, and until now no high-quality study has demonstrated a positive association between alcohol and glaucoma. In fact, it has been recognised for some time that alcohol consumption actually lowers intraocular pressure[121, 122]. The largest studies of glaucoma in the community have all demonstrated no effect of alcohol intake on glaucoma risk[118, 123-128].

Dietary Risk Factors

There is interest in dietary associations with glaucoma. Generally, the identified associations have been extremely modest in their effect size.

Amongst the more studied dietary factors, coffee and its active ingredient, caffeine, have been looked at with great interest. In the Nurses Health Study and the Health Professionals Follow-up Study (HPFS), considered by many to be the best studies addressing dietary associations to health outcomes (combined n=120,000), no effect was seen[129].

Tea, rich in antioxidative flavonoids[130], has also been the subject of interest in its relationship with glaucoma. In a cross-sectional study of 1,678 Americans, those who consumed at least one cup of hot tea per day had 74% decreased odds of having glaucoma compared to those who did not consume tea[131]. Significantly more work is required to determine if this is a potential risk modifier.

Three trials have now demonstrated examined Ginkgo biloba extract as a potential therapeutic in NTG, two of which demonstrated a protective effect particularly in field progression[132, 133] and the third demonstrated no effect on any measure[134]. Finally, a before and after analysis was used in another study and demonstrated that Ginkgo Biloba extract slowed VF progression in NTG patients[135].

The vitamin intake as determined from dietary analysis studies have demonstrated that vitamin A[136-138], vitamin B1[137], vitamin C[138]and carotenes (i.e. collard greens, kale, carrots and peaches)[136, 138] and leafy greens[139] may be protective in glaucoma. In the Rotterdam study, excluding people who take vitamin supplements showed that the biological activity of these nutrients was higher when incorporated in food rather than supplements[137]. Meta-analysis has found that dietary intake of vitamins A and C are the only reproducible vitamin findings associated with glaucoma development[140]. Conversely, one study found that participants consuming supplementary calcium or iron had significantly higher odds of being diagnosed with glaucoma[141].

Vascular Risk Factors

In meta-analysis studies, systemic blood pressure (BP) has been correlated with a small elevation in glaucoma prevalence[142, 143]. Interestingly, age appears to modify the role of BP in glaucoma prevalence, with the Baltimore Eye Study finding that systemic hypertension has a protective effect below age 60 and an adverse effect above the age of 70[144], potentially

due to the competing effect of ocular perfusion and vascular remodelling that occurs with chronic hypertension[145].

As ischemia is likely the most important pathological event in vascular approach to glaucoma, there has been much work to understand the role of ocular blood flow. A simple 'perfusion pressure' measure, calculated by the difference between BP and IOP, has been used frequently in the literature. It is now clear that lower ocular perfusion, using this measure, is strongly associated with glaucoma prevalence, and this has been demonstrated in the Baltimore Eye study[144], the Rotterdam Eye Study[146], The Barbados Eye Study[147], and the Egna-Neumarkt Study[148].

Systemic antihypertensive use has interesting and potentially paradoxical effects in glaucoma. Diastolic BP below 90mmHg due to antihypertensive use was associated with increased cupping at the optic disk, a finding that was not consistent in the untreated group with diastolic BP <90mmHg, or the treated group with diastolic BP >90mmHg[149]. The Rotterdam study also showed that people taking antihypertensive therapy were more likely to have glaucoma when their diastolic pressure was low[146]. The specific type of anti-hypertensive therapy was not shown to be important in the Thessaloniki eye study as the effect with diuretics involved in the Renin-Angiotensin System offering similar effects as diuretics or other medications, and all antihypertensive agents offered the same effects when analysed with stratified BP results[150]. However, the Rotterdam study demonstrated that Calcium Channel Blockers specifically increased the risk of glaucoma[151]. There are also other studies that have assessed antihypertensive therapies and found no effect on glaucoma prevalence[152].

Obstructive Sleep Apnoea

Obstructive Sleep Apnoea (OSA) is an illness characterised by sleep interrupted by functional occlusion or collapse of the upper airways leading to apnoea and hypoxia[153]. The recurrent apnoea leads to hypoxia (and also hypercapnia) which is responsible for this disease's complications[153].

A 2016 meta-analysis of six articles with a total of 2,288,701 participants found a strongly significant effect of OSA on glaucoma prevalence with an adjusted OR of 2.46 (95%CI 1.32-4.59) in the case-control studies and an adjusted OR of 1.43 (95%CI 1.21-1.69) in the cohort studies[154]. A registry study of the Taiwanese population found that OSA had a HR of

1.88 (95%CI 1.46-2.42) for developing glaucoma, however untreated OSA had an even higher HR of 2.15 (95%CI 1.60-2.88), whereas those with surgically treated OSA did not have a significantly elevated risk of glaucoma compared to controls[155].

Helicobacter Pylori Infection

There is a number of articles that have demonstrated that *H. pylori* infection is associated with glaucoma[156-158]. Although this has not been seen in all articles[159], a 2015 meta-analysis has shown that the finding is robust[160]. *H. pylori* eradication may also be protective in glaucoma[158] although a subsequent study could not replicate these findings[161]. A mechanism explaining these findings has not yet been established.

Socio-Economic Status and Urban Living

Socio-Economic Status (SES) is a reasonably difficult variable to measure in epidemiological research. Some studies use income[113], some use median income of household location[162], whilst others may use education level[163] and others still may use more complex indices designed on a combination of factors[164].

One of the most binary of SES classification methods is 'poverty status' (i.e. income < poverty level) which was shown to be a strong risk factor for glaucoma in the USA (OR 3.39, 95%CI 1.73-6.66)[113]. In contrast there was a positive correlation between higher income and glaucoma diagnosis in the Taiwanese population registry[165], although this may be due to access to healthcare. In the UK Biobank study, people with glaucoma were more likely to report many adverse SES variables including lower Townsend deprivation index, and income level below £18000 per year[166].

There are conflicting findings in the potential association between education level and glaucoma[113, 125, 163, 164, 167].

There is also conflicting data regarding urban residence (compared to rural residence) and glaucoma. Although a large meta-analysis found that glaucoma prevalence was higher in urban areas (OR 1.58, 95%CI 1.19-2.04), the design of the meta-analysis compared separate studies (categorising each as a rural or urban population) rather than pooling effect sizes of studies that had compared the prevalence between urban and rural populations by the same methods[17]. A Chinese study showed a similar predominance of glaucoma in the urban

population[168]. In Australian and Nigerian studies, urban residency was not significantly associated with glaucoma diagnosis[169, 170].

Obesity

The association between obesity and glaucoma is not clear[171]. A 2017 meta-analysis showed no statistically significant effect between Body Mass Index and glaucoma [172]. Central Adiposity, however, seems to be related to glaucoma and may be related to potential metabolic effects of elevated body fat. The same meta-analysis demonstrated that abdominal obesity (measured by waist circumference or waist height ratio) had a pooled risk ratio of 1.28 (95%CI 1.15-1.41) for glaucoma[172].

1.1.2 Glaucoma Management

The sole modality of glaucoma treatment remains the lowering of IOP toward a 'target level'. Since reduction of IOP is the only available treatment for glaucoma, there is almost universal utilisation of ocular anti-hypertensive agents in patients with confirmed glaucoma and patients with suspected glaucoma or ocular hypertension. Ocular anti-hypertensive medications act on different aspects of the physiology of aqueous turnover[14]. Generally speaking, most patients begin treatment with a prostaglandin analogue, due to this drug class's strong efficacy and favourable side effect profile[173, 174]. Other useful medications include beta-blockers and carbonic anhydrase inhibitors and alpha agonists and cholinergic agonists[173]. A recent review of clinical decision making in medical management investigates these with depth beyond the needs of this chapter[175].

Amongst the pharmaceutical agents, there has been some suggestion that off target effects of these may have some neuroprotective effects[176]. Alpha receptors have been found in the RGC layer[176], and animal studies have shown the IOP independent benefits of brimonidine (a commonly used topical alpha agonist) in glaucoma models[177, 178]. Beta antagonists have also been assessed for neuroprotective effect through the inhibition of calcium and sodium influx into neurons. Betaxolol, which is a more lipophilic beta blocker than timolol, has been shown to have more significant effects on slowing POAG progression compared to timolol, in small trials, despite poorer effects on IOP[179-182]. Finally, there has also been limited animal evidence to suggest that intravitreal administration of latanoprost, a

prostaglandin analogue, may have neuroprotective effects in an optic nerve transection model[183, 184]. Nevertheless, none of these has been definitively shown to have a neuroprotective effect in humans, and none of these medications are used clinically for their neuroprotective function.

When pharmaceutical management is no longer adequately able to manage glaucoma, procedural management is pursued. Procedural management for glaucoma, which includes surgical and laser options, is also aimed at reducing the IOP. Laser treatments for glaucoma offer a subjectively non-invasive procedural option for glaucoma[185-187], and in many cases, these therapies are used as a bridge between failed medical management and surgical management of glaucoma.

Surgical management is usually the last-line option for glaucoma, and involves the creation of an additional outflow tract in the anterior eye that acts as a drain for the aqueous[175]. The least radical option is the use of micro stents which, if used, are frequently placed at the time of cataract surgery[188]. Trabeculectomy is the most commonly performed surgery, involving the excision of a wedge of trabecular meshwork and adjacent corneoscleral tissue which allows for aqueous to drain into the subconjunctival space[173]. There are a number of implantable devices, referred to collectively as tubes, which drain the anterior chamber often by a tube into an external reservoir[173].

1.1.3 Pathophysiology of Glaucoma

The discovery of apoptosis and regulated cell death mechanisms significantly benefited the field of glaucoma research. The term 'apoptosis', coined in 1972 to describe controlled cell death[189], was first applied to glaucomatous cell death in 1995 by Quigley *et al.* after identifying apoptosis by the TUNEL method in axotomised RGCs in rabbits and monkeys[190]. Regulated cell death is one of the most important cellular functions in multicellular biology. There are now at least 12 regulated cell death types recognised by the Nomenclature Committee on Cell Death[191]. Within glaucoma, apoptotic cell death mechanisms are the best studied however there is some evidence that non-apoptotic cell death mechanisms such as autophagy-dependent cell death and lysosome-dependent cell death may occur[192-196]. The pathophysiological mechanisms that may contribute to glaucoma to be investigated in this thesis are inflammation and neurotrophin deprivation. A number of other pathophysiological mechanisms such as excitotoxicity and oxidative stress are also

relevant to glaucoma but are not the focus of this thesis. The mechanisms contributing to cell death should inform future therapeutic options for glaucoma.

1.1.3.1 The Role of Inflammation

The pathophysiological role of inflammation in glaucomatous cell death remains a complex issue. The causal chain linking inflammation, the immune system, and glaucomatous damage remains controversial and the subject of significant research.

Inflammatory mediators have been shown to be elevated in ocular tissues in some glaucoma research suggesting a role for local inflammation. In the aqueous humour (but not the serum) one group found that of patients with POAG had significantly elevated levels of TNFα and Interleukin-6 (IL-6)[197]. A number of studies have found associations with aqueous levels of IL-8 and glaucoma[198, 199]. Even so, each of these specific findings have failed replication in other studies[198-200]. It also should be considered that topical glaucoma medications act only after being absorbed into the anterior chamber[201], and perhaps their presence in the aqueous is responsible for the inflammatory response seen, rather than indicative of any pathophysiological mechanism. In one study that aimed to remove treatment effects as a confounding factor, ocular surface cytokine levels were seen to be different in treatment naïve eyes, suggesting that alterations to inflammatory cytokine production may still be a factor in glaucoma; although, in this particular study, cytokine levels were lower in glaucomatous eyes than in healthy eyes[202].

Systemic inflammation has not yet been linked to glaucoma[197, 203, 204]. Inflammatory cytokines may, however, interact with other signalling pathways in the retina to exert effects on RGC's, for example, IL-6 which may play a pro-inflammatory or anti-inflammatory effect, chooses its role based on the surrounding levels of Brain Derived Neurotrophic Factor (BDNF), a crucial neurotrophic factor in the retina[205]. Whilst systemic inflammation appears to be unrelated to glaucoma, intracellular inflammatory pathways seem to play an important role in glaucoma. Importantly, cellular inflammation may lead to apoptosis if pro-survival signals are overcome[206].

In animal models of glaucoma, inflammation is more readily seen, however results are difficult to interpret, as animal models usually require artificially induced tissue damage. Induced ocular hypertension in rats elevated the level of IL-1β, TNFα and IL-6 in the retina[207]. Proinflammatory cytokines (IL-1β and IL-6) were found to be elevated in the proximal optic

nerve in a DBA2/J mouse model of glaucoma[208]. Similarly, IL-1β was shown to be upregulated at a protein level after murine optic nerve crush (ONC), and knockout of *Nlrp3*, a key component of the inflammasome, was protective in this model[209].

TNFα is a cytokine that acts on the TNFR1 and TNFR2 receptors to achieve apoptosis[210]. Retinal TNFα is upregulated in human[211] and experimental glaucoma[207, 212, 213], suggesting a role for TNFα in response to RGC damage. The direct administration of TNFα to the retina leads to RGC death similar to glaucoma models[213]. Deletion of *Tnfr1* and *Tnfr2* led to RGC protection in an ONC model[214], and in an ocular hypertension model[213], respectively, suggesting targeted roles of these receptors. Glial production of TNFα may also signal RGC death through interaction with the TNFR receptors[212]. In the retina, glial cells are responsible for the majority of TNFα production, whilst TNFR1 expression is localised to RGCs[211], indicating a strong physiological direction of signalling. The release of TNFα from microglia can elicit the apoptosis of neurons through caspase 8 dependent mechanisms[215], however TNFα also potentially has caspase 8 independent mechanisms in the retina as it has been reported that caspase 8 inhibition had no effect on TNFα related cell death of RGCs *in vivo*[216].

Toll-like receptor 4 (TLR4) is a receptor on the plasma membrane involved in the signalling both inflammation and autophagy[217]. Candidate gene studies have associated *TLR4* mutations to the pathogenesis of glaucoma[218, 219]. An extreme ocular hypertension model, mimicking angle-closure glaucoma, led to TLR4 activation and formation of the inflammasome via a caspase 8 dependent mechanism in rats[220]. Subsequent work identified that HMGB1, an endogenous ligand for TLR4, is upregulated after extreme ocular hypertension, further indicating the role of inflammatory signalling in response to retinal stress[221]. TLR4 is also likely implicated in glaucomatous damage through mechanisms independent of pressure[222]. This is especially relevant since *TLR4* mutations were more commonly associated with normal tension glaucoma patients, than those with elevated pressures[218, 219]. Most members of the TLR family recognise a large number of compounds including proteins produced on microbial cell walls and damage associated molecular patterns expressed on injured or stressed cells[223]. Whilst RGCs express TLR4, TLR activation in retinal glial cells may also result in pro-inflammatory cytokine expression via the NF-κB pathway[222, 224].

It is possible that elevated intraocular pressure stimulates mechanosensitive receptors leading to intracellular inflammatory processes. Graefe's primary hypothesis

regarding glaucomatous pathology was that it was *caused* by elevated intraocular pressure[225]. Despite this, it has remained a contentious issue in the glaucoma literature if RGC's can detect mechanical stimuli. Even though, some studies have demonstrated that RGCs respond specifically to physical forces, determining the machinery responsible for converting mechanical stimulus into cellular signalling has been a difficult task, and only relatively recently have receptors with the capacity to detect mechanical stressors have been discovered in the retina[226]. Of these the best candidates are Transient Receptor Potential Vanilloid 4 (TRPV4)[227-229], the P2X7 receptor[226, 230-232], and Pannexin 1[233]. The stimulation TRPV4 leads to influx of calcium from the extracellular space[227], resulting in NLRP3 inflammasome activation[234-236]. NLRP3 inflammasome activation, through other mechanisms, has been shown as a cause of RGC apoptosis[220]. TRPV4 also interacts with Pannexin 1 and P2X7 to further upregulate NF-κB regulated transcription of inflammatory and apoptotic genes[227, 237-240]. Pannexin 1, which is a channel protein, is also involved in paracrine signalling and in the regulation of the inflammasome[241, 242]. This pathway is activated faster in cells under stress, as stressed cells downregulate gap junction proteins in favour of elevated pannexins[243, 244].

There is also some evidence that T cell activation may play a role in glaucoma. T cells have been demonstrated to infiltrate the retina in response to elevated intraocular pressure and it is possible these might contribute to glaucomatous damage[245]. A "glaucoma-related shift" has been described in the T cell subset distribution of glaucoma patients[246]. Indeed, the role of T cells is also implicated by early work in the potential use of co-polymer 1, an immunomodulator, as a potential therapy in glaucoma[247]. Clearly, much remains to be done in this area, however recent developments in the understanding of immune regulation in glaucoma are exciting.

1.1.3.2 Neurotrophins in Glaucomatous Cell Death

Neurotrophins are a family of secreted peptides that play a role in most neural processes and have a potent survival role in the central nervous system (CNS). The neurotrophin family includes Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4)[248]. Each of these neuropeptides act on a specific Tyrosine Kinase receptors (Trk) and the low-affinity nerve growth factor receptor (p75NTR)[248]. Neurotrophins are involved in virtually all neural processes in some way,

including cell proliferation, differentiation, axon and dendritic growth and neuronal morphology[248]. They are also vital molecules involved in synaptic plasticity and long-term potentiation[248]. Given the role of neurotrophins in neuron survival, which will be expanded in this section, and their potential mediation by the microbiome (discussed in '1.3.1 The Microbiome and Brain Derived Neurotrophic Peptide'), special attention is given to neurotrophins, particularly BDNF, and their role in neural biology, RGC survival and glaucomatous pathology.

Neurotrophin receptors have a well-documented antagonistic 'Ying Yang' effect is mediated through the contrasting activities of pro-neurotrophins, which have a very high affinity to the p75NTR receptor, in comparison to mature neurotrophins, which predominantly bind to the Trk receptors[249].

BDNF is initially synthesised as pre-pro-BDNF at the Golgi apparatus and is then cleaved by one set of proteases to form pro-BDNF before being cleaved by a second set of proteases to form active BDNF[248, 250]. The exact proteases and the location of these are not clear, as conflicting data exists within the literature[251-254].

The Trk receptors generally have pro-survival effects in neurons and may act by a number of different mechanisms. BDNF's effects on TrkB stimulate both the MAPK and PI3K associated pathways in the retina[255-257]. Both of these are result in neuroprotection[255]. The role of the p75NTR receptor is more difficult to understand, although its activation is highly implicated in neuronal apoptosis[258-260]. In the retina, p75NTR is only found in glia[261-263]. Furthermore, optic nerve injury leads to upregulation of p75NTR in glial cells rather than RGCs[264]. Even so, inhibition of p75NTR in Muller cells increased the survival RGC's in an optic nerve transection model[261].

There are also a number of proteins, unrelated to the classical neurotrophins, which may have neurotrophic effect[265, 266]; these are not discussed in any great detail here, except where relevant.

The Regulation of Neurotrophin Expression

The transcription of Neurotrophins is tightly regulated in the mammalian CNS[248]. The expression of these proteins has been shown to vary widely by brain sites and also with development and age[267-272]. The tight regulation of neurotrophins likely facilitates their complementary and antagonistic effects mediated through their different receptors[249].

BDNF is the most widely expressed of the known neurotrophins, and likely for this reason, it has the most complex genetics of the neurotrophin family. Human *BDNF* has at least 17 transcript variants, and 11 transcript variants have been identified in mice[273], with different splice variants being expressed in different CNS regions, and even within different cellular compartments[274]. Indeed, it has been postulated that the complex transcriptional regulation of *BDNF* allows it to have a complex range of roles, in multiple neural functions, in multiple brain regions and physiological conditions[248]. The other neurotrophic factors are less complex in their regulation with 10 transcript variants in humans (6 in mice) in total across the 3 genes[273], which, in addition to their more limited expression in the CNS[248], suggests a narrower range of effects, and therefore a narrower range of regulation mechanisms. Broadly, in the CNS, depolarization is the most significant driver of BDNF and NGF release, however a number of other factors have also been shown to cause secretion of these including hypoxia[275], microglial activation[276] and even neurotrophin signalling itself[277-280].

One aspect of neurotrophin secretion and signalling that has been of particular interest is the demonstration of endocytosis of signalling endosomes, containing mature neurotrophins, that are retrogradely transported along the axon to the soma where they may exert their effects in the nucleus[281]. The NGF-TrkA[281] and BDNF-TrkB[282, 283] partnerships both appear to propagate their trophic signal through retrograde endosomal transport of both proteins. As will be discussed, interruption of this mechanism, particularly at the optic nerve head, appears to be a feature of glaucoma.

Neurotrophins in the Retina

The expression patterns of neurotrophins in the retina help to determine the roles of these proteins in the maintenance of retinal health. It is clear that neurotrophins play an important role in the development of the retina and that the expression of these is age dependant through development[272, 284, 285]. BDNF and TrkB expression have been demonstrated in the RGCs in the adult retinae of mice[272], rats[284], zebrafish[286], and chickens[285], cats[287], dogs[288], monkeys[289] and humans[290] indicating the likelihood for a strongly conserved role for BDNF-TrkB in the protection of RGCs. BDNF is also strongly expressed at the superior colliculus (the axonal target of RGCs in the rodent brain) from where it may be retrogradely transported to the retina by the RGCs[268, 291]. The expression of other neurotrophins in the retina is not as clear[272, 292, 293].

No genetic links between *BDNF* and glaucoma in GWAS studies have been identified. One *BDNF* mutation that is of particular interest to researchers both due to its frequency in the population and its effect on BDNF physiology is the Val66Met mutation[248]. In animal models, this mutation is associated with lower extracellular concentrations of BDNF despite adequate translation of the gene[294]. In humans, the val66met mutation has been associated with distinct structural differences including reduced hippocampal[295, 296] and amygdala[296] volume in healthy individuals, increased stress-related changes in cingulate cortex volume[297], and also reduced grey matter atrophy in multiple sclerosis (MS) patients[298]. In a study of 167 Polish glaucoma patients (compared to 193 healthy controls), it was shown that the Val66Met mutation was associated with more rapid glaucoma progression, with increased rim area and cup disk ratio[299]. This study was underpowered to show an increased risk of glaucoma in Val66Met patients, however homozygous patients demonstrated a non-significant increased risk of glaucoma (OR 10.75 95%CI 0.83–138.8, p=0.069)[299]. Another candidate gene study identified that the rs2030324 SNP in *BDNF*, was associated with glaucoma diagnosis and also cup-disk ratio[300]. Addressing a genetic link in animals allows more latitude for genetic manipulation. A complete knockout of *Bdnf* in mice is generally not compatible with life, with surviving animals usually dying within the first two postnatal weeks[301]. Heterozygous *Bdnf* knockouts (i.e. expressing only one copy of the *Bdnf* gene) express half the normal BDNF protein in their various neural tissues[290]. In these mice, both age related and ocular hypertension related RGC loss was more rapid than wildtype mice[290]. Even so, more work is required to demonstrate a genetic link between *BDNF* and glaucoma.

Assessing the cellular mechanisms of neurodegenerative illnesses in humans remains difficult as biopsies of neural tissues (including and especially the retina[302]) are too invasive and dangerous to justify for research purposes alone, and therefore less direct methods are required. Serum and aqueous levels of BDNF may help to tell some of the story, as these may correlate to central levels of BDNF[303], nevertheless caution should be taken with these indirect results[303, 304]. Broadly, findings suggest that in early glaucoma, serum and aqueous humour BDNF levels are significantly lower than controls, and that this difference is less pronounced in late glaucoma[305-307]. In NTG, the level of BDNF in the tear film has been found to be as low as 1/3 the level of controls[308]. Amongst glaucoma patients, BDNF levels are negatively correlated with pattern deviation on VF[306], VF Index[307] and nerve fibre layer thickness[307]. These should be considered in light of other articles that suggest circulating

levels of BDNF mRNA and protein are not significantly different in glaucoma patients[300, 309]. One study showed that serum BDNF levels were lower in glaucoma patients about to undergo surgery (as compared to cataract patients) and that surgery led to a BDNF boost that was significant three months after surgery[310], perhaps suggesting that the success of the operation allowed for greater axoplasmic transport of BDNF to then be 'released' in the retina.

In addition to examining blood from glaucomatous patients, human disease has been evaluated by assessing tissue collected from glaucomatous eyes post-enucleation or post-mortem donor tissue. Demonstrating that a low BDNF may leave the retina vulnerable to glaucoma, optic nerve head tissues collected from post-mortem human glaucomatous eyes were shown to have a significant decrease in BDNF and TrkB protein expression compared to eyes without glaucoma[290]. A larger body of work has been performed by a group who isolated and cultured human lamina cribrosa cells and human optic nerve head astrocytes[311]. In both of these cell types expression of mRNA and protein for all four neurotrophins, and all three Trk receptors but not p75NTR, was identified[311]. It was shown that these cells have a paracrine/autocrine function, as Trk phosphorylation occurred even without exogenous neurotrophin treatment, suggesting a potential role for paracrine secretion of neurotrophins to RGCs by these cells[312]. In a subsequent study, these cell lines were exposed to oxygen and glucose deprivation, mimicking the effects of optic nerve head ischemia, demonstrating upregulation of NGF, BDNF and NT-3[313]. A sophisticated analysis of the transcriptomics of these cell lines identified BDNF as the key gene to one of three clusters of differentially altered genes shown to be altered in glaucomatous lamina cribrosa cells[314]. These findings all suggest that neurotrophin signalling from supporting cells at the optic nerve head may play an important role in human glaucoma, perhaps even through paracrine signalling to RGCs.

The literature contains many observations of animal models of glaucoma and how the retina responds to damage (animal models of glaucoma are discussed generally in '1.6 Animal Models of Glaucoma'), particularly with regards to the actions of neurotrophins. Gao *et al*. were the first group to assess endogenous neurotrophic support mechanisms in a model of glaucoma[315]. They found that after ONC in Sprague-Dawley rats, BDNF protein expression, detectable by immunohistochemistry, was significantly elevated in the RGC layer, detectable by 24 hours, peaking between 48 and 72 hours, with up to 5 fold more cells in the RGC layer expressing BDNF, and slowly declining to baseline between 1 and 2 weeks[315]. In situ hybridisation found a 54% increase in *Bdnf* mRNA 48 hours after ONC[315]. In a mouse ONC

model, a similar peak of BDNF protein expression at 3 days was found through ELISA[316]. It has been shown that the different isoforms of *Bdnf* responded differently to the ONC model, with all isoforms tested, except *Bdnf-IV*, significantly elevated by day 2 (by differing magnitudes) and remained so until at least day 7[317]. Induced ocular hypertension models have similarly been used to explore endogenous BDNF production in the eye. NGF and BDNF, and their receptors, were assessed in the retinae of rats who underwent a chronic ocular hypertension model[271]. It was seen that NGF, and its receptor TrkA were upregulated by day 7, whereas BDNF had a later peak at day 28[271]. In a similar study of ocular hypertension in rats, *Bdnf* mRNA was elevated earlier, by day 7, with maintained elevation throughout the next 8 weeks[318]. In a study of cocker spaniels, one of the few animals, aside from humans, to 'naturally' develop glaucoma, Immunohistochemistry demonstrated more intense BDNF and TrkB staining throughout the retina of glaucomatous eyes[288].

There appears to be some conflicting data in the BDNF response to ocular hypertension models. A number of ocular hypertension models of glaucoma have demonstrated no model induced response of BDNF[316, 319-321], including one group whose ocular hypertension results contrasted to the BDNF response they saw in response to ONC[316]. These results suggest a more complex, or unclear, relationship between ocular hypertension and neurotrophin support.

There is also extensive evidence that retrograde transportation of neurotrophins is interrupted in experimental models of glaucoma in primates[322-325] and rodents[289, 326, 327]. Furthermore, in humans, analysis of enucleated specimens has shown that there is significant axonal transport failure in the RGCs of people with secondary glaucoma[328]. Glaucoma-like disc changes and VF defects have also been described in people with intracranial tumours[329], and it has been suggested that this may be due to a mass effect causing a defect in axonal transport of neurotrophins through the optic nerve in these patients[330]. Abnormal focal labelling for BDNF and Trk receptors were identified in the axons at the optic nerve heads of glaucomatous monkeys[289]. Indeed, many animal models have demonstrated that increased IOP can lead to interruptions in the axoplasmic flow of neurotrophins to the retina[288, 289, 331, 332]. As RGCs project to the superior colliculus, is it is an interesting finding that both chronic and acute ocular hypertension models[333] as well as ONC[316] cause a significant rise in the BDNF of the target neurons at the superior colliculus. This upregulation of BDNF occurs before the loss of RGC synapses suggesting that it is an endogenous stress response mechanism to

prevent some RGC death[333]. The manifestation of a reduction in target derived BDNF in RGC survival is delayed for some months, after ablation of the target tissue, where RGCs project to in the CNS[334, 335]. It is likely that local production of neurotrophins by stressed neurons in the retina may compensate for the target derived factor deficits[336].

Interruption of a number of pathways in the process of RGC degeneration has been shown to cause increased BDNF production and signalling. It remains unclear for many of these pathways if their interruption is relevant to the neuroprotective effects of neurotrophins, however, these processes merit some examination. siRNA mediated knockdown of Optineurin was associated with downregulation of 112 other genes including *Bdnf* and this disruption in the gene's role was associated with an increase in apoptosis in this model[337]. siRNA targeted against a mTOR inhibitor was shown to elevate the levels of neurotrophins NGF, NT3 and BDNF and also increase cell survival in a rat ONC model, demonstrating that collateral gene regulation is an important aspect to gene regulation with definite functional outcomes[338]. Interruption of oxidative stress with phosphine-borane complex significantly elevated BDNF in the retina, and had a retinal protective effect that was TrkB dependant[339].

BDNF as a Therapeutic Agent in Glaucoma Models

Interventional studies have made up the bulk of neurotrophin related glaucoma research. Of this, the vast majority of the research has focused on the role of BDNF. The first published study to inject BDNF into the eyes of animals in a glaucoma model appears to be from 1994[340]. In that study, female Sprague-Dawley rats who underwent optic nerve transection were also given an intraocular injection of BDNF, leading to no significant loss of RGCs after 1 week (compared to a loss of 50% in untreated controls), and at the end of 2 weeks surviving RGCs were still significantly greater than untreated controls[340]. Soon after this, BDNF was shown to significantly protect RGCs in a more gentle ONC model, although the complete preservation of RGCs at 1 week was not replicated[341]. Similar results have been replicated numerous times in various models[342-348]. Other neurotrophins have also shown some neuroprotective benefit but, BDNF consistently provides superior neuroprotection to RGCs when compared to other neurotrophins[349-353].

Interestingly, BDNF treatment, whilst effective for the prevention of total RGC loss, failed to have a neuroprotective effect on the small subpopulation of melanopsin-expressing RGCs in the retina[354].

The levels of BDNF in the brain may also be relevant to glaucoma. Weber *et al*. compared cats after ONC who had received no neurotrophic intervention with cats who received either a single intravitreal injection of BDNF or a combined treatment of an intravitreal injection and continuous delivery of BDNF to the visual cortex via brain infusion pump[355, 356]. At 1 week after ONC, a single intraocular injection of BDNF improved RGC survival from 55% to 79%, and at two weeks from 31% to 60%, which is comparable to previously published work in other animal models[356]. However, the combined application group of cats (who received both the intraocular injection as well as an intracerebral infusion of BDNF) had statistically indistinguishable in RGC survival compared to no crush at 2 weeks (with an average of 92% RGC survival)[356]. In an extended study with the same study protocol, 4 weeks after ONC RGC survival was 7%, 29%, and 53% in the control, injection only and combined therapy groups, respectively[355].

There are a number of limitations of BDNF as a therapeutic agent for glaucoma. Firstly, BDNF administration to the retina usually requires an intravitreal injection which is of limited clinical tolerability in an illness that is slow and mostly asymptomatic. For this reason some groups have begun looking into other administration options such as BDNF eyedrops, which in one study were comparable to intravitreal injection[348]. Another limitation of intravitreal injections of BDNF is that injections of higher concentrations of BDNF initiate inflammation in the retina, which had a small but measurable negative effect on RGC survival[357], putting a limit on the neurotrophic effect that can be expected by injections of exogenous BDNF. BDNF injection also causes a reduction in the retinal expression of TrkB protein, which may limit the efficacy of BDNF injections, or multiple injections, for the protection of damaged RGCs[358].

Recent advances in interesting delivery methods have been presented in the literature. One area that has shown promise is the use of nanoparticles to deliver neurotrophins to the optic nerve, of which both NGF and BDNF have demonstrated efficacy through this method[359]. The use of nanoparticle delivery has resulted in a significantly lower dose of neurotrophin required to protect RGCs, and these lower doses when given as free BDNF or NGF showed no protection capacity[359].

Aiming to offer prolonged BDNF support, gene-therapy has also been used by numerous groups to increase BDNF and protect neurons in animal models of glaucoma, predominantly through adenovirus-associated vectors (AAV). The use of an AAV-BDNF vector to upregulate the mRNA and subsequent protein expression of BDNF in primary retinal neurons led to slower apoptosis rate in culture[360]. Amongst *in vivo* models, AAV vectors introducing *Bdnf* to the retina has shown benefit in chronic ocular hypertension model[361], ischemia/reperfusion[362]. Of the cells in the retina, AAV vectors appear to be most effective in raising Müller glia expression of BDNF, which may then signal to RGCs[363]. The tamoxifen-cre recombinase model, an alternative to AAV gene therapy, and has been used to boost BDNF in an ocular hypertension model and ONC, resulting in the protection of RGCs[364, 365]. In models with low magnitude of BDNF response to AAV vectors, these vectors are not particularly beneficial[366, 367]. Similarly, despite being the target of the optic nerves projection, AAV-BDNF administration to the superior colliculus, rather than the retina, did not demonstrate protection of the RGCs[316].

Stem cell therapy may also result in BDNF signalling in glaucoma models. Intravitreal injections of stem cell have demonstrated benefit in excitotoxic retinal damage[368] and ONC[369, 370]. These stem cell injections result in upregulation of BDNF in these models[368, 369], and their neuroprotection appears to be TrkB dependant[370]. Combining techniques of stem cell applications and genetic therapy to upregulate BDNF has been successful in some trials. In a chronic ocular hypertension model, the incorporation of engineered *Bdnf* expressing mesenchymal stem cells offered significantly greater functional outcomes and cell survival than those rats who were given *Gfp* expressing stem cells[371]. Another study found that Sprague-Dawley rats, who had undergone ONC, and treated with *Bdnf* expressing neural progenitors had significantly higher RGC survival than those who had undergone the same ONC treated with genetically normal neural progenitors, and sham controls[372]. Finally, Hu *et al.* recently used stem cells genetically modified to interfere with endogenous neurotrophin signalling, finding that inference with both BDNF and glial-cell derived neurotrophic factor (but not the presence of genetically normal stem cells) increased the RGC loss in ONC demonstrating the endogenous role of both of these in normal neural homeostasis[373].

Pharmacotherapeutics Acting on the Neurotrophin Pathway

Aside from direct administration of BDNF, a number of therapeutic options have been assessed for their role in modifying neurotrophin pathways including TrkB agonists, some immunotherapy agents and the off-label use of established medications.

Agonists for BDNF's key receptor TrkB have been suggested as a method to drug this pathway. 7,8-dihydroxyflavone, a TrkB agonist[374], protected primary RGCs from excitotoxic damage and oxidative stress[375]. Other groups have shown similar protective effects of 7,8-dihydroxyflavone in other neuronal cell lines[376, 377]. A meeting abstract also suggested it played a protective role in an ocular hypertensive model of rat glaucoma[378]. Groups have also developed monoclonal antibodies that activate TrkB. The 29D7 antibody protected RGCs both in primary cell culture and an *in vivo* optic nerve transection model, with similar efficacy to BDNF[257]. The 1D7 monoclonal antibody delayed RGC death in both optic nerve transection and an ocular hypertension model of experimental glaucoma[379].

Brimonidine, an older glaucoma medication used for reduction of IOP through alpha2 receptor agonism, was shown to elevate the BDNF of RGCs at a mRNA and protein level in 'normal' rats[380]. Subsequently, it has been shown that a brimonidine injection protects rats from ONC initiated retinal degeneration, which is likely to be independent of its pressure reduction effects given that ONC is a pressure independent model[178].

Valproate, an anticonvulsant medication has been shown to acetylate histone K3K14 in the *Bdnf* promotor, promoting transcriptional activity of *Bdnf*[381]. Valproate protected RGCs in a rat ONC model through the activation of BDNF mediated pathways[381]. Valproate has also demonstrated similar effects in excitotoxic retinal degeneration, mediated by upregulation of BDNF in Müller cells[382]. In a placebo controlled pilot study, 3 months of oral valproate was associated with improved visual acuities in glaucoma patients[383]. Butyrate, a microbiome metabolite which is also able to act on histones in the *Bdnf* promoter, also had similar effects to valproate in RGC degeneration models[381, 384].

It had been shown previously that transference of activated T cells to Myelin Basic Protein protected RGCs in a model of ONC initiated in rats. These T cells, which express neurotrophins NGF, BDNF, NT-3 and NT-4/5, when transferred to rats demonstrated a significant Trk receptor dependant pro-survival effect[247]. Along these lines, copolymer-1 (also known as glatiramer acetate) is an immunomodulator, with biochemical similarities to myelin basic protein, normally used in the treatment of MS, may also offer some benefit. Copolymer-

1 protected RGCs in a rat model of ONC through a protein kinase C and BDNF mediated mechanism[385]. Two further studies, with combined injections of stem cells with copolymer 1 demonstrated neurotrophin upregulation and increased cell survival in ONC[386] and ocular hypertension[387]. The benefits of copolymer-1 immunisation require further investigation as another study was unable to see any copolymer-1 related increases in NGF, BDNF or NT-4 in the rat retina[388].

1.2 The Microbiome

There has been a radical shift in our understanding of what it means to be human. In recent years, it has become increasingly clear that each human plays host to trillions of microbes that inhabit every surface of our bodies creating complex multi-kingdom ecosystems. The microbiome, a word that refers to the total contents of the microbial community that live in and on a host, may contain bacteria, archaea, eukaryotes (including fungi and other eukaryotes formerly referred to as protists) and viruses. The networks that these microbes form are complex, highly plastic, habitat-specific (i.e. differ by location on the host), and are finely tuned to adapt to changes in host physiology. We are only beginning to understand their role in health however their extensive effects are increasingly seen.

Often cited, to an almost clichéd extent, is the suggestion that the human microbiome is made up of approximately ten times as many cells than human cells in an adult human host. A recent reassessment of this suggested that the ratio of microbes to human cells in an adult is probably closer to 1:1[389]. Nevertheless, landmark sequencing studies have demonstrated that the collective genome of the human microbiome contains at least 2-20 million genes, compared to the ~20,000 of the human genome[390, 391], suggesting that the microbiome makes up a huge proportion of the genetic diversity of an individual.

One of the most conclusive truths demonstrated in the last fifteen years of genomic research is that genetics provide only a limited explanation of the variation in disease. As we now know, this is the case with even the best-studied illnesses only achieving moderately better prediction models with the inclusion of genomic data (see Muller *et al*.'s comprehensive 2016 review[392]). However, these findings demonstrate the profoundly significant role of non-genetic causes of illness, often referred to as environmental causes. If we consider the human as a separate entity from the microbiome, then it is possible that the microbiome is the most important environmental factor in determining human health[393, 394]. Moreover, if we consider the microbiome as a part of the individual, as an integral part of the 'holobiont', then the microbiome represents the most substantial interface between which the individual and the environment interact. The microbiome, like the human genome before it, represents a resource that remains largely undocumented, yet full of promise for increasing understanding of health and disease.

1.2.1 The Normal Microbiome

With the rise in understanding regarding the relevance of microbiome in disease, there has been interest in determining what is considered a normal or healthy microbiome. Most of this research has targeted the colonic microbiome as the gut contains the largest component of the microbial biomass in a human[395].

Sequencing of the microbiome, like sequencing of the human genome before it, was a revolution in the understanding of the make-up of the microbiome. By sequencing of the 16S ribosomal subunit gene, taxonomic understanding of the components of the microbiome is now possible (described in Figure 1.2). Primarily it became clear that the human microbiome has very high level of interindividual variation[390, 391, 396] and therefore that a 'normal' microbiome made up specific and standard taxa that would be found in all healthy individuals was an unlikely hypothesis. In fact, only approximately a third of an individual's microbiome gene pool is shared by most humans[390, 397]. Furthermore shared taxa have been found to vary beyond an order of magnitude in their abundance between individuals[396].

In addition to the 16S subunit sequencing utilized in the characterization of the constituent microbes of the microbiome, 'shotgun metagenomics', which involves sequencing the total DNA of the sample and linking the sequences with the functional capacity of the genes, has also played an essential role in understanding of the functional make-up of the microbiome (also described in Figure 1.2). The data produced by the Human Microbiome Project hinted that the microbiome contained a functional core, rather than a taxonomic core[391]. This functional core is assumed to have metabolic and other functions that are reproducibly performed by the microbiome, but different taxa may be responsible for these in different individuals[398]. The functional core must include the functionality required to maintain microbial life, however, the functionality that may form the basis of symbiotic, mutualistic behaviour is of particular interest[395].

The MetaHIT cohort, which assessed the microbiome of 124 healthy individuals, identified an estimated 1000-1150 bacterial species that could be found in a human of which each person carried approximately 160 species[390], however this number is limited by the resolution of the sequencing and other research has claimed that the average person may have up to 1000 species of bacteria residing within their gut[399].

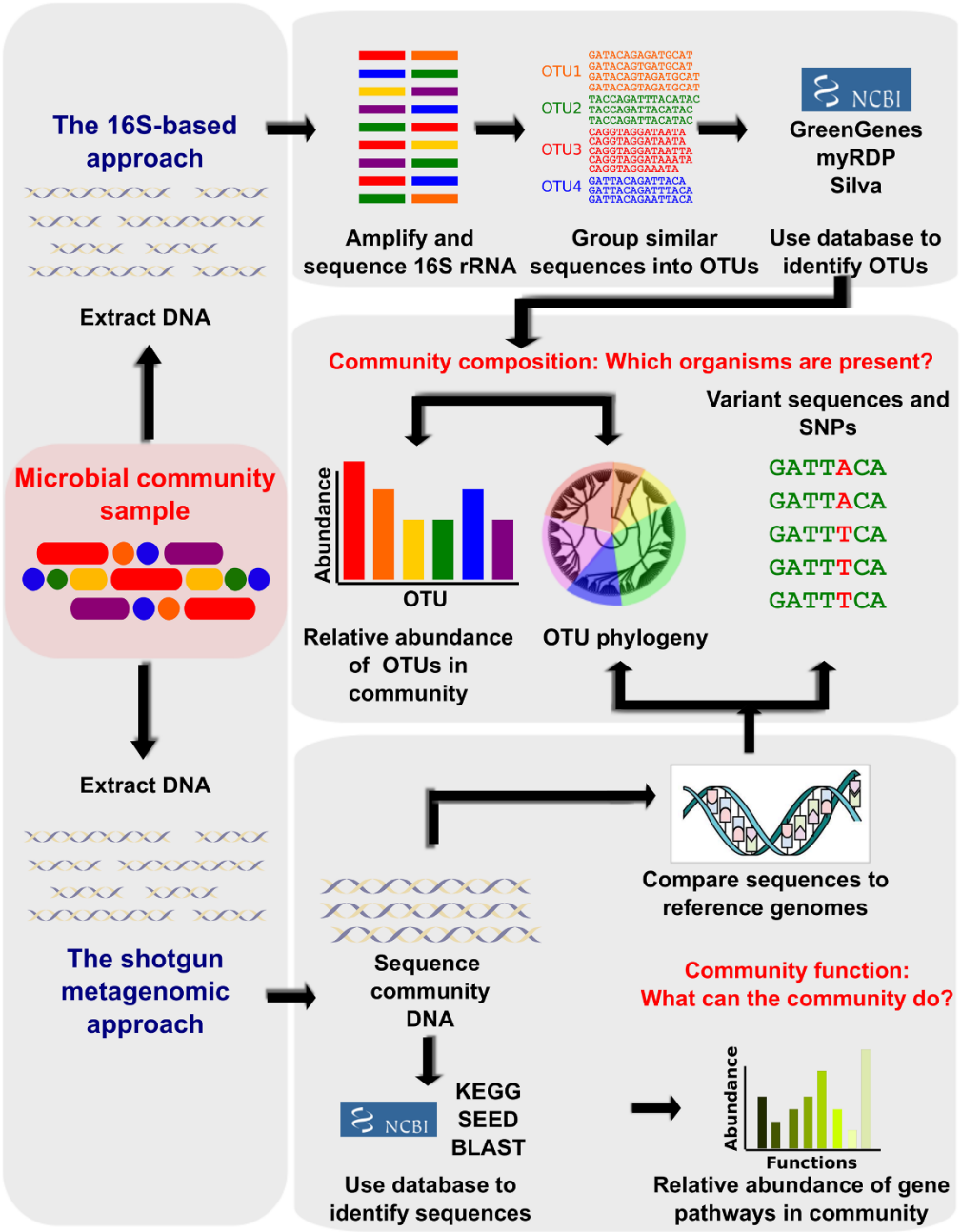


Figure 1.2: Methods for microbiome metagenomic analysis

Firstly, DNA is extracted from a microbial community containing multiple microbial members. The bacterial community members are frequently defined by amplifying the 16S rRNA gene and sequencing it. DNA sequences are grouped into Operational Taxonomic Units (OTUs), which can then be compared to databases to precisely identify their taxonomy. The primary alternate method of identifying community taxa is the shotgun metagenomic approach. Community DNA is directly sequenced and compared it to reference genomes or gene catalogues. The functional capabilities of the community can be determined by comparing the sequences to functional databases. This allows the community to be described as relative abundances of its genes and pathways. It may also be used to identify taxonomy of the microbiome, with greater specificity as this method captures single nucleotide polymorphisms (SNPs) and other variant sequences. Figure reproduced according to the CC-BY-NC 4.0 license from Morgan *et al.* PLoS Comput Biol 2012:8;e1002808[400]

The first and most obvious is the exposure of the infant to the vaginal and faecal flora as it exits the birth canal. Whether or not this transmission is particularly important for the development of the microbiome is unclear. It is known that the microbiome of infants differs depending on mode of delivery[401], but conflicting results have been published regarding the length of time this difference persists[402-406].

After birth, there is further vertical transmission through colostrum and milk, which may supply between 8,000 and 800,000 bacteria per day[407]. The withdrawal of milk and the introduction of solid food is associated with a decrease in *Bifidobacteria*[408]. The richness of the microbiome grows rapidly through this period[409-411], and it is clear that the immune system rapidly develops during this period; it's also highly likely that the development of these is significantly related to the development of each other[412, 413]. Animal models which demonstrate that successional colonisation of microbes is dependent on the prior colonisers[414], suggesting that disturbing the microbiome in development may have significant knock-on effects.

Puberty heralds the gender specification of hormonal signatures and is the period where one develops a gender-specific microbiome[415]. The microbiome of adolescents has been shown to be distinct from adulthood, suggesting that even further microbiome development occurs as an adolescent matures[409, 416].

By the time a person becomes an adult, their bacterial microbiome exhibits more stability compared to early life[408]. Generally speaking, changes to the microbiome during adulthood usually represent a change in an environmental exposure, such as diet[417] or antibiotics[418-420]. Beyond adulthood, further aging leads to changes in the microbiome[411, 421] although these studies by definition suffer from survivor bias. Determining the role of an ageing microbiome on health is an area that remains largely unexplored.

Other body locations are less well studied than the gut; however, it is clear that various anatomical locations on the host contain highly specific microbial communities[391, 395]. The oral microbiome is similar in complexity to the gut microbiome, however with a lower biomass[391]. The skin microbiome is also fairly complex and differs according to the regional skin differences[391, 395]. It is more difficult to analyse the microbiome of body locations with very low microbial biomasses, however these (including the lung, breast milk and placenta, and potentially blood and intra-tissue) represent microbial communities of interest for their relationship to health[395].

When considering the relationship between the microbiome and eye disease, it is worth mentioning the ocular surface microbiome. The legitimacy of a true resident ocular microbiome has been a controversial topic, until recent years, due to the minimal apparent bacterial presence in the tear film[422]. The sterile ocular surface argument probably stems from the low rate of culture positivity of ocular surface swabs, however, the fact that these swabs usually have no growth even in established bacterial ocular surface infection[423] suggests that culture techniques are inadequate to capture this information. Microbiome sequencing has revealed a significant breadth of bacteria that may be found on the ocular surface, and has established that the ocular surface microbiome is associated with geographic residence and ethnicity[422], and also the use of contact lenses[424, 425]. These 'demographic' specificities suggest that the microbiome of the ocular surface may be true residents, more than simple contamination. Moreover, the independence of the ocular surface microbiome to other microbiomes including nearby skin microbiomes[424, 426] also limits the likelihood that it simply represents contamination. Additionally, as determined by sampling of conjunctiva from eyes undergoing pterygium surgery, there appears to be a 'crypt' associated microbiome (in the conjunctival folds at the fornices and limbal crypt-like structures known as Palisades of Vogt) that is significantly different to what is demonstrated on ocular surface swabs[427]. This crypt associated microbiome appeared to be specifically dominated by *Pseudomonas* (~80%, whilst being only 6% of the surface microbiome) whereas the ocular surface microbiome is more varied with *Corynebacterium*, *Streptococcus*, and *Serratia* dominating at much lower abundances (between 5-15% each)[427]. Both the ocular surface and the 'crypt' microbiome contained roughly 4-7% Acinetobacter, and 2-4% Thermoanerobacterium[427].

By sheer number, bacteria do not represent the most numerous members of the microbiome. That honour belongs to the viral component of the microbiome, made up predominantly of bacteriophages[428], which is likely to outnumber the bacterial component by a factor of 10 to 1[428, 429]. Understanding how the normal human virome functions may be beyond our capabilities at this stage given that the great majority of purified samples are not related to any currently identified viruses and the rate at which viruses evolve is rapid[428]. Furthermore, the viral component is even more interpersonally variable than the bacterial component of the microbiome[430, 431]. It is likely that both through its action on the bacterial component of the microbiome, and direct actions on the host, demonstrated by emerging

symbiotic interactions between viruses and the hosts[432, 433], the viral component of the microbiome will be seen to play an increasingly important role in human health and disease[428].

Though they make up only a tiny proportion of the microbes in the gut, the presence of archaea in the microbiome has been known for some time, as these were some of the early culturable members of the microbiome[434]. The *Methanobrevibacter* genus is the most common archaea genus seen in the microbiome[435] and their H_2 consumption activity likely promote the health of other members of the microbiome[436]. As there are only a few recognised archaea residing within the microbiome[436], their role in health has not been the subject of much research. However, one group has begun looking at certain members of these as potential probiotic agents[437].

Traditionally seen as pathogens, the role of eukaryote microbes in the microbiome and their effects on health is increasingly investigated[438]. Both beneficial and (apparently) commensal eukaryotes have been identified including *Saccharomyces boulardii* which has been used as a probiotic to treat diarrheal illness[439]. Fungi and *Blastocystis* are often the only eukaryotes in the microbiome of a healthy individual, with a relatively low diversity noted[438, 440]. Outside the gut, the skin is known to host a fungal community dominated by *Malassezia*[441] and the oral microbiome contains a more complex fungal microbiome[442], it remains to be seen how these impact health[438].

Given that the microbiome is made up of thousands of species spanning the tree of life[390, 391, 443], determining the 'health' of the microbiome is particularly difficult. At this point in time, studies aimed at identifying a healthy microbiome have focused on identifying the features of the microbiome inhabiting "healthy individuals who lack overt disease phenotypes"[395]. Sequencing studies using these definitions have shown that the definition of a healthy microbiome is complex, and does not seem to present as a binary outcome. Considering the complexity of the bacterial portion of the microbiome is already immense, adding these other domains of life further complicates analysis. To further understand the concept of a healthy microbiome, the concept of the holobiont may help.

1.2.2 The Holobiont and Hologenome Theory of Evolution

The 'microbiome' together with their host form the 'holobiont'. 'Holobiont', a word initially coined by Lynn Margulis to refer to a host and their endosymbiont[444], was adapted to the complex range of life that lives within coral[445], and has come to refer to any plant or

animal and the totality of their respective microbiome[446]. The holobiont (and its associated 'hologenome', the comprehensive set of genes associated with the host, its organelles, and the total microbiome) has since become the focus of evolutionary biologists, suggesting that a holobiont likely acts as a unit of selection in evolution, giving rise to the hologenome theory of evolution[446-448].

A microbe and its host can theoretically exist in a relationship characterised anywhere on a symbiotic continuum from parasitic to commensal to mutualistic[449]. Indeed, there is a rich literature on the existence and evolutionary development of binary symbiotic relationships of both parasitic and mutualistic nature. Hologenome theory rests on the assertion that the microbiome-host interaction in its entirety and its complexity must be mutualistic and that the health of the whole organism is disrupted when then symbiosis is disturbed[446, 447, 450]. Moreover, a breakdown in the symbiosis in the holobiont better defines 'dysbiosis' than merely a shift in the constituent microorganisms.

Although not particularly philosophically engaging, it could be argued that the purpose of life is to procreate. Evolution is the process that occurs when advantages (beneficial phenotypes) gained by an organism are passed on to subsequent generations. It appears that microbes have been on earth for around 3.5 billion years[451], with the evolution of multicellular organisms only taking place within the course of the last 600 million years[452]. It is therefore implied that multicellular organisms have always evolved in the presence of microbes and it is expected that multicellular organisms that tolerated or even benefited from microbes were the most likely to survive.

The hologenome theory asserts that the holobiont, made up of the macrobe host and its multitudinous microbial symbionts, is a unit of biological organization[446-448, 450]. Further, macrobes have always been holobionts[450, 453]. Simple genotype by environment analysis (where the host and microbiome could play either role) taken to either extreme does not recognise the evolutionary capacity or complexity of the other partner. Determining how these interact therefore should focus on genotype (host) by genotype (microbiome) by environment interactions, where the host and microbiome interactions may form a sort of intergenomic epistasis[450].

The holobiont appears to be a comprehensive mutualistic system where both the microbiome and the host benefit from the relationship. It is clear that microbes in a holobiont exhibit smaller genomes than similar microbes not in a symbiotic relationship[454] suggesting a

selection for the elimination of redundancy in the symbionts whose generational cycles are much shorter than their more complex hosts. Moreover, the closer the relationship between the symbiont and the host, the smaller the genomes, for example, obligate symbionts have a smaller genome than facultative bacteria which in turn have a smaller genome than free-living bacteria[449, 454]. Host resource utilisation should drive genome reduction in symbionts; similarly, the environmental specialisation that occurs when a symbiont takes residence inside the niche of a host should lead to redundancy in genes that confer survival in the environment[449, 454]. One example of this is that the constituent microbes of a microbiome residing inside a host rapidly lose the genes that help them to cope with UV radiation[449].

Symbionts may provide the host with the genetic diversity that may increase their fitness in new circumstances. Cute examples of this are the obligate bamboo eating Giant Pandas (*Ailuropoda melanoleuca*), which despite their diet, lack the genes to produce cellulose degrading enzymes, and consequently have evolved with a microbiome that fills this functional void[455]. Furthermore, members of the Carnivora order (which includes the Giant Panda) are deficient in a critical cyanide detoxifying enzymes, which presents a problem for obligate bamboo eaters, as bamboo is abundant in cyanide-containing compounds, yet evidence now shows that Giant Pandas microbiome is enriched in genes encoding for cyanide degrading enzymes[456]. Their microbiomes ability to cope with these demands is considered, alongside the morphological adaptations Pandas have undergone (such as pseudothumbs), as an important evolutionary process that has enabled the Giant Panda to survive[456]. It is unlikely that processes like these do not exist in humans, maintaining the health of the holobiont through functionality provided by the microbiome.

The nullifiable hypothesis central to the holobiont theory is that host genes, and components of the microbiome, are co-inherited to the degree that evolution can operate in their interaction. A more recent development in holobiont theory is the development of the idea of phylosymbiosis. Coined by Brucker and Bordenstein in 2013[457], phylosymbiosis refers to the idea that the microbiome differs increasingly as host phylogeny does. This idea suggests that despite the significant variation in the microbiome, its development is still deterministic rather than stochastic and therefore its 'inheritance' still plays a role in evolutionary processes. The logical inference of phylosymbiosis, in light of the hologenome theory, is that the host selects its microbiome in a manner to achieve inter-hologenomic epistasis and that if either is significantly disturbed, deleterious effects may occur[457].

There is some evidence that the western lifestyle, may break down the photosymbiotic relationship between humans and the microbiome. In a study that compared the microbiome of adults in Papua New Guinea (PNG) to those from the USA, whilst demonstrating more diversity than their US counterparts, the microbiome from those in PNG was shown to be less individualized[458]. This raises the question that perhaps the western lifestyle is responsible for an uncoupling of typical phylosymbiotic relationships in the human microbiota, and therefore an increased dysbiotic burden in the west.

The hologenome theory of evolution is an important tool for understanding how macrobes have evolved in a world overrun with microbes. Providing an evolutionary basis for understanding the mechanisms that lead to colonisation and formation of the microbiome within the host provides important ideas on how the holobiont integrates to protect its health. Dysbiosis, therefore, becomes more of an evolutionary concept relating to the health of the current organism and all potential offspring.

1.2.3 Defining Dysbiosis in the Context of the Holobiont

Considering the microbiome's importance within the hologenome, and therefore its importance not just to the present health of an individual but also its effect on evolutionary processes, the current widely used definitions of dysbiosis are woefully inadequate. Most studies investigating the role of the microbiome in health and disease simply state that the microbiome from a person with no overt signs of disease is representative of a healthy microbiome[395], calling any taxonomic or functional differences in the microbiome associated with the malady of choice, dysbiosis[459]. However, it is well known that significant differences in the human microbiome accompany diet[417, 460-462] and geographic location[458, 460, 463-465], neither necessarily indicating an alteration in the health of an individual. In fact, the pathophysiological relevance of a previously identified 'dysbiosis' associated with diabetes mellitus has now been called into question as the effects of metformin on the microbiome explain some of the previously identified differences in 'diabetic microbiome'[466].

Reasons for the difficulty in defining a microbiome signature considered 'healthy' come from hologenome theory. As the concept of phylosymbiosis contends, the makeup of the microbiome and its relevance to human health and evolution does not necessitate vertical inheritance of a specific set of organisms. As the deterministic processes that help the holobiont to select and cultivate the microbiome are due to functional interactions between

the host and the symbionts (and within the symbiont community), neutrality (functional equivalence of various microbes) will lead to stochastic variation in the deterministically acquired microbes[450]. This likely explains why animals, including humans, have a core microbiome when judged from functionality[391, 467] or higher levels of phylogenicity[390, 391, 467] but not at lower phylogenetic levels, such as species.

To simplify understanding of the normal or healthy microbiome, Shapira proposed a layered approach to the microbiome[468]. Simply put, the microbiome contains a 'core' strongly associated to the host, likely responsible for the majority of mutualistic and beneficial interactions within the holobiont, and a 'shell' made up of facultative symbionts semi-randomly acquired from the environment. It is entirely possible for microbes to shift in their status from obligate symbiont to facultative symbiont (or vice versa), in fact for at least one species of bacteria, the shift in symbiotic lifestyle has occurred multiple times in its evolutionary history[469]. If the core is considered in a similar way to the functional component of the host genome (which incidentally, despite ENCODE estimates of 80% functionality[470], has recently been suggested to be a lot smaller than previously thought[471, 472]) and the shell considered to be like 'junk DNA', varying in much the same way that neutral genes do, offering only rare phenotypic significance, then the understanding of the microbiome becomes more sound. It also forces one to reconsider the common definition of dysbiosis.

The technically correct definition of dysbiosis must be limited only to the description of a breakdown in holobiont symbiosis. Symbiosis within the holobiont is maintained by a number of different host factors, particularly the immune system, and also by the interactions within the microbiome. Hologenome theory suggests that the different components of the holobiont, when challenged by expected stressors in their environment, should adapt in the best interests of the whole holobiont thereby ensuring stability within the system. Dysbiosis is the breakdown in the homeostatic mechanisms and represents a destabilisation in the holobiont. Dysbiosis is therefore characterised by a change in the functional capacity of the microbiome that leads to deficits within the holobiont. Stability of the microbiome as defined by the microbiomes capacity to resist a disturbance, and its resilience after disturbances are also important descriptors of the microbiomes health[395, 473]. Dysbiosis can occur due to host factors, extrinsic environmental factors, or the interactions of both of these.

Peterson and Round[459] proposed that three primary mechanisms contribute to what they called dysbiosis; "loss of beneficial organisms", "expansion of pathobionts" and "loss of diversity"[459]. These mechanisms offer a framework for the present investigation into dysbiosis.

High diversity in the microbiome has been generally associated with health[459]. Microbial diversity acts as the principal source of genetic variation within the holobiont. Diversity has been associated with resilience and stability of the microbiome[474]. The relationship between these suggests that functional redundancy is achieved by having multiple constituents, with differing tolerances to different insults, performing the same function; termed the insurance hypothesis[473]. The comparative loss of diversity seen in the microbiomes of those from developed nations has been hypothesised to play an important role in the increased burdens of chronic diseases seen in these nations compared to the developing world[475, 476]. The 'western diet' is one of the lifestyle factors that has been blamed for the 'the disappearing microbiome' seen in developed nations[475, 476].

Whilst the majority of associations made to diversity indicate its beneficial nature, it is important to note that increased diversity is not always beneficial. Elevated microbiome diversity in the gut microbiome has been associated with Autism Spectrum Disorder[477], Major Depression[478] and HIV infection[479]. Similarly, in other microbiomes, higher diversity in the vaginal microbiome has been associated with bacterial vaginosis[480] and cervical neoplasia[481].

Fundamentally, though, it should be noted that diversity metrics offer little information regarding the functional significance of the constituents[482]. Therefore, whilst they may be helpful in some circumstances, a numerical diversity score offers only a limited understanding of the health of the microbiome. Fundamentally, alterations in diversity (in either direction) implicate further research to understand the functional implications of these changes.

The holobiont may also be harmed through loss of beneficial organisms, whether it be loss specific microorganisms with close microbe-gene interactions, or through loss of certain functions that may be maintained by multiple different microbes. Underpinning this may be feedback loops involving the host's immune system and community destabilisation. Within the context of the holobiont, loss of beneficial symbionts should provide a similar effect as loss of function mutations within the host genome, however identifying specific beneficial bacteria may be difficult within the complexity of the total microbiome.

Some beneficial host-microbe interactions occur between partners that are specified even below species level, as demonstrated by the symbiosis of *Caenorhabditis* species and their specific Enterobacteriaceae symbionts[483]. Although it is reasonable that host microbe interactions at this highly specific level may occur in humans, determining the species or strain is immensely difficult in the noise of big-data experiments.

It is clear that some factors may lead to long-term alterations in the microbiome that may lead to the depletion of beneficial symbionts. A long term study that examined the effects of antibiotic usage demonstrated a strong shift in the microbiome to the point where differences in the microbiome were detectable at the end of follow-up[418-420, 484-487]. The causal pathway through microbiome modification in illnesses where antibiotics are a risk factor (asthma[488-490], other atopic diseases[490], inflammatory bowel disease[491-493], and perhaps type 2 diabetes mellitus[494]) has not been fully investigated.

Redundancy of function, influenced by host factors such as diet, where genes required to metabolise certain dietary compounds are unnecessary due to their absence from the host's diet, may also be a source of beneficial microbe loss. In much the same way that a genome may undergo reduction to remove redundant genes, the microbiome may be reduced when parts of it become functionally redundant. One major example of this is the absence of a set of bacterial genes for cellulose and xylan hydrolysis in European children despite their relative abundance in children from Burkina Faso[460]. Interestingly, the reversible loss of diversity that accompanied the feeding of humanised mice fed on a western diet became fixed after just four generations[495] which demonstrate the rapid ability for the microbiome to adapt to the environment of the host and eliminate redundant functionality. It is likely that microbes, not required by the host, become functionally neutral, going dormant, and are minimised until they are eventually, perhaps after several host generations, lost through drift.

Whilst the net effect of the microbiome is beneficial, there are certain microbes, with pathogenic potential, who present a problem for the host if circumstances allow[496]. These microbes, termed pathobionts, may still contribute to health, so their activity and usually their predominance, and not their existence, is what is considered harmful[497]. In humans, members of Proteobacteria, particularly members of the Enterobacteriaceae family, are the most commonly identified pathobionts[459] and their association with inflammation and colitis is well known[498, 499]. However other bacteria may also be considered as pathobionts such as *H. Pylori*,

known to cause stomach cancer, but found in approximately half the world's population[500]. The conditions suggested to be important to its pathogenicity are host factors such as mutations in the immune system[501, 502], and perhaps bacterial dysbiosis in the surrounding microbiome[503]. Although pathobionts may seem like the optimal target for developing definitions of dysbiosis, the decades of work looking to identify organisms responsible for inflammatory bowel disease and other illnesses (especially in the years after Marshal and Warrens Nobel winning discovery of *H. Pylori*'s relationship to peptic ulcer disease) suggests that these are only, at most, part of the picture[459]. Therefore, definitions of dysbiosis must rely on more than simply the identification of 'bad bacteria'.

A healthy microbiome, in addition to its 'current functional capacity', must be stable. Stability of an ecological system as a measure of its health was first described by Holing, and these principles can be applied to the microbiome[504]. Stability can be assessed in two ways; firstly, a healthy microbiome should have a substantial ability to withstand disturbance, termed 'resistance'; secondly, the healthy microbiome should, after an insult significant enough to disturb it, rapidly return to a healthy state, termed 'resilience'. It is clear from longitudinal studies of the human microbiome that despite its interindividual variability there is significant long-term stability in its make-up[391, 505]. It is also important to note that, whilst an unstable microbiome represents a dysbiosis, an unhealthy microbiome may achieve a stage of relative stability and resilience, which may lead to long-term health impacts on the host and therefore stability alone does not define dysbiosis.

A number of factors that contribute to the assembly of the microbiome have been identified[506], of which selection (i.e. the fitness dependent changes in the microbiome due to the needs of the holobiont) is most relevant to microbiome resilience[507]. Selection can occur both externally (i.e. the hosts impact on the microbiome) and internally (i.e. the interactions between the microbes). In addition to selection, the role of microbial dormancy is important in synthesising diversity with resilience. It has become clear that many constituents of microbial communities can become dormant when the current environments are not especially conducive to metabolic activity[508]. These remain in the microbiome, albeit non functionally, but may become reactivated as the environmental conditions change[509]. Interestingly, at least in other microbial populations, rarer microbes were more likely to be active than more abundant microbes in the community[510]. In the human microbiome, members of the Firmicutes phylum are more likely to be active than members of the

Bacteroidetes phylum[511, 512]. Microbial dormancy has been suggested as a way for the microbiome to maintain functional potential without the metabolic cost of maintaining microbial activity[510].

In summary, defining dysbiosis in the context of the holobiont requires identification of microbiome states that reduce the health and fitness of the holobiont. Compromise of the beneficial components of the holobiont, suggested by a loss of beneficial symbionts or more broadly by the loss of diversity, represents a loss in the functional capacity of the holobiont and therefore a reduction in fitness and health. The expansion of pathobionts presents deleterious changes within the microbiome thereby impairing the health of the holobiont. Finally, loss of stability within the microbiome indicates that the microbiome is unable to cope with the homeostatic demands of the holobiont. The concepts of the holobiont, therefore, provide ample justification for understanding why the microbiomes constituents do not necessarily determine its health but only sustained phenotype altering disturbances of the microbiome represent true dysbiosis.

1.3 The Microbiome and the Central Nervous System

Since the seminal work by Sudo *et al*., published in 2004[513], there has been a growing body of literature demonstrating an association between the Microbiome and CNS processes. These findings are particularly interesting as the CNS has been considered a privileged system protected by the Blood Brain Barrier (BBB), for most of the contemporary era. The most consistently differentially expressed proteins identified in the CNS in response to microbiome alteration is BDNF, however, there is also growing evidence that the microbiome plays a role in the maintenance of the BBB and the neuroimmune system. On top of this, there have been clear inroads in the assessment of the microbiome in neurodegenerative illnesses.

1.3.1 The Microbiome and Brain Derived Neurotrophic Peptide

Sudo *et al.*, reasoning that as there is rapid development in the immune system and the Hypothalamic-Pituitary Adrenal (HPA) axis in early life, and that these have significant cross-talk, the microbiome, which programs the immune system, may play a role in the neural networks associated with the HPA axis and stress response[513]. Through a number of studies using Germ Free (GF) BALB/c mice, they became the first group to demonstrate that the resident microbiome is involved in the programming of the HPA axis, particularly in response to stress[513]. Whilst their findings focused on the stress response, in particular, on corticosterone and Adrenocorticotropic hormone production in response to restraint, they discovered interestingly, that in the cerebral cortex and the Hippocampus of GF mice, there was a significantly lower level of BDNF protein at rest[513]. They also demonstrated that monocolonising GF mice with a pathogenic bacteria (Enteropathogenic *Escherichia coli*) or a probiotic bacteria (*Bifidobacteria infantis*) worsened or obviated, respectively, the 'GF' effect on the HPA axis[513], suggesting that the effects of microbiome alteration could be rescued even in adulthood.

The understanding of the relationship between the microbiome and BDNF greatly expanded in 2011 with several large research projects investigating this relationship with a much broader scope. Neufeld *et al*. investigated the anxiety-like behaviour patterns of GF Swiss Webster mice, correlating these to genetic patterns in the hippocampus. They found that GF mice had reduced anxiety-like behaviours than specific pathogen free (SPF; normal microbiome) mice and that this correlated to a lower NMDA receptor subunit NR2B mRNA in the amygdala as well as increased BDNF in the dentate granule layer of the hippocampus[514].

Diez Heijtz *et al*. also assessed the anxiety behaviour in GF mice, however, the focus of their investigation was synaptic plasticity markers in the brain[515]. GF NMRI mice demonstrated reduced anxiety-like behaviour with increased motor activity, which correlated to increased monoamine turnover and increased expression of synaptic proteins, synaptophysin and PSD-95, in the striatum, suggesting a significant increase in the activity in this brain region in GF mice[515]. They also demonstrated that *Bdnf* mRNA expression in the cingulate cortex, the hippocampus and the amygdala was significantly reduced in GF mice[515]. Bercik *et al*. attempted to reveal the mechanism for the interaction between the microbiome and BDNF expression changes in the brain[516]. Using antibiotics to induce changes in the microbiota, this group found that disturbing the microbiota similarly decreases the anxiety-like behaviour of BALB/c mice and that this was reversible with normalisation of microbiome[516]. Oral antibiotics were associated with decreased BDNF in the amygdala and increased BDNF in the hippocampus[516]. Interestingly this group found that the same antibiotic treatment in mice who had been given a vagotomy or a sympathectomy both has similar results in the assessment of behaviour as mice without autonomic neural interruption suggesting that the gut-brain effect is mediated by something other than autonomic nervous system communication[516] (though, a recent study demonstrated that vagotomy in adult SPF mice leads to decreased *Bdnf* mRNA expression in the hippocampus[517], implying that the model in GF mice should be revisited). Finally, Bercik *et al*. found that the colonisation of GF BALB/c mice with microbiota from NIH Swiss mice led to BALB/c mice presenting with an NIH Swiss-like phenotype in behavioural testing and BDNF levels in the hippocampus[516]. Similarly, colonisation of GF NIH Swiss mice with BALB/c microbiome demonstrated opposite results[516], together indicating that specific patterns of the microbiome may exert specific neuronal phenotypes.

GF studies offer the cleanest demonstration of the effects of the microbiome removing the specific off target effects of any microbiome altering agent from the analysis. In a study of the social development of GF mice, GF mice exhibited altered social behaviour when interacting with an unknown mouse. This behaviour was theorised to be related to the amygdala, and therefore the expression of *Bdnf* was assessed in the amygdalae of these mice and found to be significantly lower in GF mice than SPF mice.[518] In an altogether different study, Schele *et al.* assessed the role of GF status on the body-fat regulating centres (the hypothalamus and the brain stem) finding that in these locations *Bdnf* mRNA was significantly

higher in GF mice than conventionally raised mice[519], which contrasts with the majority of the other literature on this topic. They also found that *TrkB* mRNA was significantly higher in the brainstems of these mice[519].

Significant alterations of the microbiome, induced by antibiotic treatments, offer external validity to real-world biology, where the host may encounter environmental challenges but will never develop in any sterile manner. Antibiotic-induced dysbiosis of NIH Swiss mice initiated at weaning (i.e. adolescence) was shown to cause similar anxiety behaviour phenotypes as maintenance of GF status[520]. Similarly, these mice had reduced BDNF in the hippocampus[520]. *Bdnf* mRNA reduction in the hippocampus as well as the prefrontal cortex, and the hypothalamus has also been shown in antibiotic depletion (by a slightly different cocktail of antibiotics) of adult C57BL/6 mice[521]. Antibiotic-induced dysbiosis (by yet another antibiotic cocktail) was shown by Guida *et al*. to downregulate BDNF protein expression in the hippocampus, whilst upregulating TrkB expression. BDNF expression, but not TrkB expression, was normalised in dysbiotic mice who were treated with a *Lactobacillus casei* probiotic[522]. Altogether these results provide good evidence to support the notion that the effects seen in GF mice translate to physiologically typical situations.

There are a wide variety of stimuli that have been shown to alter the levels of BDNF in the brain that may act by indirect microbiome effects. Stressful stimuli are known to alter microbiome in animal models[523, 524]. Restraint stress led to lower *Bdnf* mRNA expression in the hippocampus of rats, this was recoverable with treatment of a *Lactobacillus helveticus* probiotic[525]. Prenatal stress on pregnant animals led to altered microbiome of their offspring, and also decreased BDNF in the amygdala, which may be due to prenatal exposure of the foetus to elevated levels of IL-1β[526]. Dietary modulation, in addition to providing a separate source of nutrients to the host also shifts the microbiome in semi-predictable ways[417, 527]. The 'western diet' leads to decreased BDNF expression in the hippocampus[528, 529]. The similar 'high fat diet' also impairs BDNF levels in the hippocampus, with functional correlation, and this has been shown to be recoverable with probiotic feeding[530, 531]. Finally, and perhaps most interestingly, given the relationship between aging and glaucoma, aging has been shown to lead to reduced levels of BDNF in the brain, which in more than one study was reversible with probiotic supplementation[532, 533].

1.3.2 The Microbiome and the Neuro-Immune System

The immune system's core function is control of tissue damage. Historically and evolutionarily, one of the biggest causes of tissue damage was infective organisms and for this reason, given that the microbiome appears at first glance to be infecting the host, without negative sequelae or overt immune activation, the majority of microbiome research has focused on the interactions between the microbiome and the host immune system. It is now abundantly clear that the microbiome is responsible for the maturation of the immune system[413]. GF mice demonstrate defective maturation in the gut-associated lymphoid tissue, including reduced numbers and sizes of Peyer's patches, lower numbers of mesenteric lymph nodes, and impaired development of the lymphoid follicles[413, 534]. GF mice also show defects in the T helper cell populations[535] and T reg cells[536], and in the populations of circulating phagocytes including neutrophils[537] and macrophages[538]. For reviews on the systemic breadth of microbiome immune interactions see Maynard *et al.*[413] and Thaiss *et al*[539].

The immune system is active in the CNS, and the microbiome has been shown to have important effects on neuroimmune function. Data is accumulating to suggest that the microbiomes effects on both the innate immune system[540, 541] and the adaptive immune system[542, 543] may play a role on the path of neurodegenerative illnesses.

Microglia are the resident macrophages of the CNS, a core part of the innate immune system, responsible for defence of the CNS from pathogenic attack. Microglia are also responsible in part for tissue repair, however abnormal activation of these cells is being increasingly seen as a cause of psychological and neurological illness. When microglia are activated (which may occur by a wide range of stimuli, including the detection of microbial molecules[544]) they begin to secrete molecules that have proinflammatory effect in the tissue, further recruiting more microglia, with the intention to clear the source of the stimulus that activated these cells to begin with[545]. The inappropriate activation of these cells and the corresponding release of proinflammatory peptides, results in damage to healthy neurons and through this mechanism contribute to neurodegenerative processes[546].

Maturation of microglia clearly requires a functional microbiome. GF mice have defective microglia, whereby there are broadly immature compared with SPF mice and had significant defects in their capacity to respond to a normal stimulus[540, 541]. Lipopolysaccharide (LPS) stimulation (a model of bacterial infection) and viral infection both resulted in blunted responses in GF microglia compared to SPF microglia, including a significantly lower

production of proinflammatory cytokines in both models[540]. Absolute numbers of microglia were higher in GF mice, and their morphology was significantly different to SPF microglia both before and after LPS stimulation[540]. This group also found that antibiotic-induced dysbiosis resulted in similar microglial phenotypes to GF microglia and that conventionalisation or Short Chain Fatty Acid (SCFA) treatment of GF mice rescued the phenotypes displayed[540]. Demonstration of the stepwise maturation process of microglia by a subsequent group[547], has gone some way toward explaining these findings. In this study, the investigators found that the differences in the microglia of GF mice were significantly more pronounced in adult animals compared to newborns and therefore that there was a defective pathway in the maturation from pre-adult to adult phenotype[547].

Another group noticed that the immaturity of the microglia was, at different stages in development, more pronounced in a sexually dimorphic manner[548]. Abnormal microglial cells were more 'abnormal', as determined by RNA-Seq and morphology, in male GF embryos compared to female GF embryos, and more abnormal in female GF adults compared to male GF adults[548]. Furthermore, the treatment of adult mice with antibiotics leads to sexually specific alterations in the responses of microglial cells[548].

Dysfunction of microglia (or analogous cells in invertebrates) has been associated with a number of neurodegenerative phenotypes in microbiome research in animals[541, 549]. Microbiome effects have been especially clear in Parkinson's Disease (PD). Sampson *et al*., using a genetic knock in model of PD, noticed that in animals whose microbiome had been depleted, microglia were immature with defective proinflammatory response[541]. This poor inflammatory response correlated to an attenuated Parkinson's phenotype, both of which could be 'rescued' with SCFA supplementation[541]. Another group found that the resident microbiome of Parkinson's mice were enriched for SCFA production (compared to wild-type), and that if these mice received the microbiome of wild-type mice, inflammatory cytokine production and microglial activation in their brains was significantly attenuated[550]. In a drosophila model of Alzheimer's Disease (AD), increasing the abundance of two separate pathobionts led to increased neurodegeneration via a TNF mediated neuroinflammatory response[549]. Immune haemocytes (functional macrophages of flies) were more readily recruited to the brains of the dysbiotic flies, and hemocyte depleted flies demonstrated that this recruitment was required for neurodegeneration[549].

In the last couple of years, it has become clear that the microbiomes effects on the adaptive immune system particularly T cells also has drastic effects on the CNS phenotype of an individual, particularly in response to insult.

Two studies have found that mice humanised with microbiome from MS patients have worse outcomes in experimental autoimmune encephalomyelitis (EAE; a murine model of MS) demonstrating that the MS microbiome specifically increases the phenotype of the autoimmune insult[551, 552]. Both groups found that these effects seemed to be regulated by IL-10 expressing T cells[551, 552].

In a study of mice undergoing Middle Cerebral Artery Occlusion (MCAO) model, antibiotic-induced microbiome shift led to increases in regulatory T cells, a reduction in IL-17$^+$ γδ T cells in the intestines, and defects in the normal trafficking of IL-17$^+$ γδ T cells to the meninges after stroke[542]. Interestingly this effect was associated with a reduction in infarct volume, which was attributed to the reduction in neuroinflammatory cellular activity associated with the deficiencies in IL-17$^+$ γδ T cells[542]. In a study of genetically identical mice from different breeders, it was shown that the relative abundance of segmented filamentous bacteria in the microbiome correlated well with the ratio of regulatory T cells and IL-17$^+$ T cells[543]. It is likely through this mechanism that the abundance of segmented filamentous bacteria was correlated well to infarct volume when these mice underwent MCAO[543]. Further linking the adaptive immune system to the microbiome's effects in stroke pathology, lymphocyte depletion obviates the difference between GF and SPF mice in MCAO outcomes[553]. Spychala *et al*. assessed the role of microbiome aging in systemic inflammation and then how this may play a role in neurodegenerative processes in stroke[554]. Using faecal transplants young and aged microbiome were assessed for their ability to impact on host MCAO responses, demonstrating worse outcomes in mice with aged microbiomes, due to proinflammatory effects[554].

1.3.3 The Microbiome and the Blood Brain Barrier

The BBB is a vital protective structure in the CNS of animals. It facilitates the protection of the brain and central nervous system from potentially toxic chemicals that may be found in the circulation. Whilst inflammation and BDNF signalling are more likely to be involved in a pathophysiological effect of the microbiome on glaucoma, the BBB merits assessment, as its functionality could theoretically limit microbiome-CNS communication.

There is some evidence that the Microbiome regulates the BBB[555]. Increased BBB permeability, in GF mice compared to age matched SPF mice, begins in utero (in the foetuses of GF mothers) and is maintained until adulthood, and this was associated with reduction in tight junction proteins, Occludin and Claudin-5[555]. Conventionalisation of these mice was shown to rescue to some extent the permeability demonstrated in GF mice[555]. Finally, a probiotic treatment (both *Clostridium tyrobutyricum* and *Bacteroides thetaiotaomicron*) of GF mice was shown to Increase the effectiveness of the BBB indicating that specific microbes can have a significant effect on the BBB[555].

Although no clear replication has been performed, some of these findings have been seen in other studies. Occludin and claudin-5 were variably expressed in certain regions of the brains of antibiotic-treated mice; in the hippocampus occludin and claudin-5 were downregulated in antibiotic-treated mice, in the amygdala of these mice occludin was elevated alongside the mRNA for another BBB protein, tight junction protein-1; no changes were identified in the hypothalamus or the frontal cortex[521]. Low dose penicillin V treatment in dams 1 week prior to birth until weaning to initiate dysbiosis in the pups of BALB/c mice[556]. In this study, the pups demonstrated increased levels of occludin and claudin-5 in the hippocampus but not the frontal cortex[556]. Finally, to contrast the initial findings discussed in this section, antibiotic induced microbiome shift had no effect on the BBB permeability in mice undergoing stroke, neither before nor after the initiation of a stroke model[542]. Even so, sodium butyrate (a microbiome metabolite) is known to inhibit the activities of histone deacetylase which has preservation effects in ischemia-mediated breakdown of the BBB[557].

1.3.4 The Microbiome in Neurodegenerative Disease

As glaucoma is a neurodegenerative illness, the microbiomes contribution to other neurodegenerative illnesses has been assessed for the purposes of identifying relevant pathways for the analyses performed in this thesis. What follows is a brief survey of the literature for each of the major neurodegenerative illnesses, highlighting the key findings for each.

1.3.4.1 Alzheimer's Disease

AD is caused by the deposition of amyloid plaques that results in the death of neurons, leading to atrophy of the brain tissues beginning in the hippocampus[558]. Given that many of the previously identified microbiome-brain interactions occur in the hippocampus, the impacts of the microbiome on AD are biologically plausible.

A few recent studies have now shown that the gastrointestinal microbiome of people with AD is significantly different to healthy controls. Vogt *et al*. found that the richness and alpha diversity (richness and abundance of the taxa within a sample) of the faecal microbiome were reduced compared to controls in a cohort of Caucasians with AD[559]. Similarly, significantly different community profiles were identified on beta-diversity analyses[559]. Correlations between specific genera and cerebrospinal fluid (CSF) levels of beta amyloid was seen demonstrating a linear correlation between certain microbiome elements and AD pathology[559]. A similar but larger analysis was performed in a Chinese population also finding distinct microbial communities in the AD group compared to the healthy group[560]. Neither study assessed the potentially confounding role of anti-Alzheimer's medications on the microbiome. An indirect study of the microbiome was performed by another group who found that IgG's to bacteria resident in the oral microbiome differed between AD and healthy populations, potentially linking microbiome and AD through immunological pathways[561]. Using an hypothesis-free computational modelling on large datasets of metabolite, gene and protein interactions, as well as databases of profiles of AD patients, it was demonstrated that microbial metabolites are likely to play a role in AD pathology[562].

The assessment of post-mortem brain tissue has been valuable in assessing the penetrance of the microbiome to the brain. In a study of post-mortem brains, AD patients were seen to have double the LPS in the neocortex and triple the LPS levels in the hippocampus, of non-AD patients[563]. This compares to elevated levels of rhamnolipids (bacterial virulence factors) seen in the CSF of AD patients[564]. A separate group sequenced the bacterial DNA of post-mortem brains, identifying that intracerebral bacteria were more numerous and significantly different from the intracerebral bacteria seen in non-AD post-mortem brains[565]. Obviously questions remain about the potential for contamination however the timing between death and tissue processing time did not correlate to bacterial load[565], suggesting that tissue processing was less likely to cause contamination, and previous

studies have identified the brain and the blood may host bacteria (or at least bacterial DNA) in non-septic patients[566, 567].

There is also significant indirect evidence that microbiome alteration is contributory to AD including relationships between AD and both diet and IBS. In a meta-analysis of 5 studies, those who were most adherent to a Mediterranean diet had a reduced risk of Mild Cognitive Impairment and AD compared to those least adherent to the diet[568]. Recently published research shows higher representation of Bacteroidetes and a lower Firmicutes–Bacteroidetes ratio are found in individuals who maintain a higher adherence to the Mediterranean diet[569], perhaps explaining the beneficial nature of this diet on the CNS. Beyond diet, it was shown that Irritable Bowel Syndrome (IBS) increased the risk of non-Alzheimer's dementia with (aHR 1.24, 95%CI 1.15-1.33) and Alzheimer's (aHR 1.76, 95%CI 1.28-2.43) particularly after the age of 50 in a Taiwanese cohort[570].

Animal studies have readily shown that genetic defects that contribute to the development of AD may shift the microbiome[571-576]. It is important to note that causality is difficult to establish in these studies, as, for example, the intestines of AD mice expressed greater levels of Aβ Precursor Protein which may account for why genetic models of AD may be associated with altered microbiome[575]. Addressing the causality issue, GF $APP^{swe}/PS1^{\Delta E9}$ mice exhibited brain Aβ protein levels 57% and 70% lower than the levels seen in conventional AD mice at 3.5 and 8 months, respectively, which could be 'rescued' when GF mice were reconventionalised[571]. Similarly cerebral amyloid deposition was reduced in the GF mice in both the cortex and the hippocampus, and this was also rescuable[571]. Another study used broad-spectrum antibiotics to induce dysbiosis in $APP^{swe}/PS1^{\Delta E9}$ mice to show that long-term shifts in the microbiome decrease Aβ plaque deposition but increase the load of soluble Aβ suggesting, in this model, that the microbes impact on a plaque formation pathway[577].

If dysbiosis plays a role in AD pathophysiology, speculative treatments such as probiotics or dietary administration of microbiome metabolites may play a role AD therapy. In one study a probiotic mix of *Lactobacillus* and *Bifidobacterium* strains ameliorated memory and learning deficits in an intra-hippocampal Aβ injection model of AD, perhaps through oxidative stress related mechanisms[578]. A 12 week placebo controlled randomised control trial (RCT) of a probiotic mixture in 60 AD patients noted that probiotic supplementation led to an improvement on the MMSE of 27.9%, compared to a loss of 5.0% in control subjects[579],

although long term follow up was not performed. These improvements were correlated to circulating C reactive protein (CRP; an inflammatory marker) and also to levels of circulating malondialdehyde (a marker of oxidative stress)[579]. Recently the same group published findings that there appears to be a critical window of severity within which probiotic therapy offers the most benefit to AD patients[580]. The use of dietary administration of microbial metabolites has demonstrated some effectiveness in both a *Caenorhabditis elegans* model[581] and a mouse model[582, 583] of AD. These findings though are far from conclusive but do lend further evidence toward the microbiome neurodegeneration theory.

1.3.4.2 Parkinson's Disease

It is well known that early PD is associated with gastrointestinal upset and that this may manifest as constipation or other bowel symptoms[584]. Furthermore Braak *et al*. has shown that PD pathology, although best known for its substantia nigra pathology, is first seen in the CNS in the dorsal motor nucleus of the vagus nerve (which connects the enteric nervous system to the brain)[585]. In fact, it has been shown in a retrospective case series that alpha-synuclein is present in the enteric nervous system (assessed through biopsy specimens in colonoscopy patients) up to 5 years prior to the development of PD symptoms[586].

Cross sectional analyses have demonstrated significant differences in the microbiome of people with PD when compared to healthy controls, in various populations from around the world[587-592]. One study demonstrated that microbiome profiling can discriminate, with reasonable specificity, people with PD from healthy controls, and certain symptoms have been linked to the abundance of specific microbes[588]. Another study also found that specific taxa were associated with motor subtypes of PD, although microbial composition overall was not[593]. Idiopathic REM sleep behaviour disorder has been considered as a prodromal phase of PD[594], and therefore it has been used as a comparison cohort for PD. In a faecal microbiome study, 80% of the differentially prevalent microbes in the stool samples of PD compared to healthy controls were similarly different in idiopathic REM sleep behaviour disorder patients suggesting that the microbial changes associated with PD are an early feature of the illness[595]. Beyond the gut, the oral microbiome has also been shown to have some relationship to PD diagnosis[596], but the nasal microbiome was a poor differentiator of PD from control patients[596].

Importantly, PD medications are associated with specific patterns in the microbiome, with signatures noted to be associated with catechol-O-methyltransferase-inhibitors, anticholinergics, and possibly carbidopa/levodopa[597]. To combat this another group specifically, assessed the microbiome of PD patients prior to treatment to protect their analysis from medication-induced changes[598]. Whilst specific alterations in certain taxa were noted, on a functional assessment, they found that PD microbiomes exhibited altered ß-glucuronate and tryptophan metabolism, and interestingly they also had decreased virus abundance[598].

Beyond microbiome community analysis, quantification of the products of the microbiome has also been useful in demonstrating the effect of the microbiome in this illness. Faecal SCFA concentrations were lower in faeces from PD patients[592], and specific urine-excreted metabolites of the microbiome were found to be significantly higher in PD patients than in healthy control patients[599].

Longitudinal assessment of the microbiome has shown that specific alterations to the microbiome may lead to worsening symptoms of PD over time. In a 2 year study of 36 patients, low abundance of *Bifidobacterium* and *Bacteroides fragilis* was associated with worsening Unified Parkinson's Disease Rating Scale and worsening psychiatric symptoms[600]. This study was underpowered, however its findings indicate the microbiome may be used to predict the course of PD. These results were justified by a subsequent placebo controlled RCT, of 60 PD patients, assessing the beneficence of a probiotic mixture containing *Bifidobacterium bifidum* and three *Lactobacillus* species[601]. After 12 weeks, the probiotic group had an average improvement of 4.8 points on the Unified Parkinson's Disease Rating Scale whilst the placebo group worsened by 3.8 points[601]. This also correlated to CRP levels which were found to be improved in probiotic patients but not in placebo-treated patients[601].

Given that many people with PD have clinically significant constipation prior to typical neurological symptoms[584], it is very likely that gastrointestinal function changes (such as decreased motility), which could plausibly be triggered by neural pathology alone, will lead to statistically significant microbiome changes that are unrelated to any true dysbiosis. Even so, an interesting finding of one paper was that the level of constipation was not a significant covariable for microbiome composition in a cohort of PD patients and controls[595].

The most apparent evidence for the role of the microbiome in PD comes from the substantial article published by Sampson *et al*. which demonstrated the role of the

microbiome in a mouse model of PD[541]. In their study, they found that the presence of the microbiome potentiated the development of both fine motor and gross motor deficits in a mutant mouse model of PD[541]. GF mutant mice similarly had reduced α-synuclein aggregation compared to SPF mice, demonstrating that microbiome presence potentiates synucleinopathy[541]. The effects seen may be mediated by poor activation of the neuroimmune system as microglial immaturity was identified in the GF brain[541]. Motor deficits in SPF mice could be minimised by 'depleting' the microbiome with broad-spectrum antibiotics, and GF mice could be made to have a PD phenotype with colonisation with microbiome from SPF animals at weaning, demonstrating the role of postnatal signalling in this pathway[541]. Similarly, in GF mice, SCFA treatment was sufficient to induce synucleinopathy, and associated symptoms, in mutant animals[541]. Finally, the group colonised GF mutant mice with human microbiome from PD patients and healthy controls demonstrating that PD microbiome in humans causes greater motor dysfunction in mutant mice than healthy microbiome[541]. Altogether these results signify that the microbiome is relevant in the development of PD.

1.3.4.3 Multiple Systems Atrophy

Multiple systems atrophy (MSA) is the name given to a spectrum of rare and fatal neurodegenerative syndromes due to α-synuclein inclusion bodies aggregating in oligodendrocytes (in contrast to the build-up of α-synuclein inclusion bodies in neurons in PD)[602, 603]. There have been two studies that assessed the microbiome in patients with MSA. Engen *et al*. collected faecal microbiome and sigmoid colon biopsies from 6 MSA patients and 11 healthy controls[604]. Assessing the mucosal biopsies showed that MSA was associated with disruption of Zonula-Occludens structure and increased inflammatory markers in sigmoid mucosa suggesting intestinal barrier dysfunction[604]. Faecal and mucosal microbiome both demonstrated significant different alpha diversity between the two groups with effects also seen for specific groups of microbes[604]. Imputed functionality assessment found that the microbiome of MSA patients, both in the faeces and the mucosa, was more capable of producing LPS[604]. A conference abstract reported on 17 MSA patients compared to 17 controls[605]. Specific microbes were noted to be different in MSA patients[605]. Clearly, in a rare illness, it will be difficult to interrogate the microbiome with the finesse that has been

achieved in PD, however, given that both illnesses are synucleinopathies, there is potential that progress in researchers understanding of microbiome in PD may translate to MSA.

1.3.4.4 Multiple Sclerosis

Despite the fact that MS is not often considered to be a neurodegenerative illness, there is convincing evidence that neurodegenerative processes are an important component of its pathology[606-608]. Even so, as its mechanisms are primarily immune, its relationship to microbiome is only partially relevant to glaucoma.

Generally speaking, the community structure of the microbiome in MS patients is similar to that of healthy controls. Most microbiome profiling studies have not found community level microbiome differences. Standing out from the rest, two studies have suggested broad community differences between MS and healthy microbiome[609, 610] (though in one of these, a later re-analysis of the data with different statistical modelling, called into question the findings displayed[611]). At lower phylogenetic levels, effects were seen in all[551, 552, 612-616] but one study[617]. Generally speaking, the microbiome differences displayed between active MS, MS in remission and healthy controls, is only seen when looking at specific microbes or specific groups of microbes.

Nevertheless, these findings demonstrate the issues with microbiome profile analysis as despite the general finding that community structure is similar between MS patients and healthy controls, when the microbiomes from patients are transferred to animal models, the effects on phenotypes are remarkable. In two of the aforementioned studies, microbiome from MS patients and their controls were transferred into mice MS models of experimental autoimmune encephalomyelitis (EAE) demonstrating that the MS microbiome specifically increases the phenotype of the autoimmune insult[551, 552]. In one of these studies the severity of the EAE phenotype was able to be associated with the abundance of specific species of microbes[551]. In the other of the studies, microbiome from five monozygous twin pairs, discordant for MS, was transferred into GF mice from a strain that undergoes spontaneous EAE[552]. In their model, mice colonised with MS microbiome developed spontaneous EAE at a much higher rate than those colonised with the microbiome of the healthy twin[552]. Both microbiome transfer studies found that microbiome mediated effects seemed to be regulated by IL-10 expressing T cells[551, 552]. Interestingly, it has been shown that microbiome are

necessary to cause EAE as it is not seen in GF animals[618], and modifying the microbiome of resistant animals increases susceptibility[619, 620].

Beyond the gut, there has also been some question as to whether the microbiome may translocate into the circulation and then travel to the CNS where they may directly be able to cause inflammation. Branton *et al*. sequenced the 16S rRNA of autopsied brains from MS patients and age and sex-matched controls identifying a 'brain microbiome', dominated by Proteobacteria and Actinobacteria in all samples[621]. These findings were backed up by immunohistochemistry and in situ hybridisation of samples to demonstrate the presence of bacterial DNA and bacterial proteins in all brains[621]. Interestingly the total bacterial burden was not different between MS patients and controls[621]. In contrast to this study, Jovel *et al*. performed a sequencing study on the CSF of MS patients and controls identifying significantly more bacterial DNA in controls than in MS patients[622]. Bacterial DNA was only detectable, at a rate of >1% of reads in one of 28 MS patients compared to eight of 15 controls[622].

1.3.4.5 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS), also known commonly as motor neurone disease, is a neurodegenerative illness that affects the motor neurons, of the brain and the spinal cord, that control the voluntary muscles[623].

A disease-causing variant in the *SOD1* gene has been transferred to mice to investigate ALS. Although these mice are asymptomatic until after 3 months of age, microbiome alterations and gastrointestinal mucosal deficits are detectable by the age of 2 months[624]. Microbiome defects and lifespan, were improved in animals who had been treated with a butyrate solution[625].

In humans, there have been three articles published assessing the make-up of the microbiome in ALS. A study of 25 ALS patients (23 sporadic cases), found that diversity and abundance of bacterial taxa, compared to 32 age gender-matched controls, were virtually indistinguishable[626]. Inferred functional analysis of the microbiome (PICRUST) were also very similar[626], together indicating to the authors that ALS patients do not exhibit a substantial alteration of the gastrointestinal microbiome[626]. Nevertheless, in a proof of concept study, Rowin *et al*. suggested that the microbiome of 5 ALS patients was dysbiotic as they had decreased diversity and lower Firmicutes/Bacteroidetes than a (much larger) control population[627]. In the largest of the studies, qPCR based methods revealed a higher abundance

of *E. coli* and enterobacteria and a lower abundance of total yeast in ALS patients[628]. PCR denaturing gradient gel electrophoresis analysis allowed for differentiation of the profiles of ALS patients from healthy controls[628].

1.3.4.6 Stroke and Ischaemic Brain Damage

Ischaemic brain damage is the second largest cause of mortality worldwide, and in patients who survive, there is a significant burden of neurodegenerative morbidity. Stroke associated secondary neurodegeneration is an increasingly understood cause of long-term morbidity in ischemic brain disease[629]. This slower neurodegenerative process usually occurs in locations in the brain that are connected to the infarcted zone but are not completely infarcted themselves, often termed the penumbra. These secondary neurodegenerative processes occur due to ischemia-reperfusion induced inflammatory cascades amongst other processes[630]. For this reason, mechanisms in stroke damage may have particular relevance to glaucoma, another illness whereby there is a progressive loss of neurons due to stress factors.

There are a couple of studies that have looked at the microbiome in humans to assess the role of the microbiome in stroke. Yamashiro *et al*. assessed the faecal microbiome of 41 ischaemic stroke patients and compared this to the microbiome of 40 healthy controls[631], finding differences in the abundance of specific microbes, and systemic inflammatory maters[631]. In a smaller study, there were significant differences in the abundances of certain genera in the microbiomes of cases and controls[632]. The biggest issue with cross-sectional microbiome research in humans is that the stroke cohort's microbiome is sampled after the stroke and therefore may be impacted by the stroke pathology or its treatment. Animal studies have consistently shown that a stroke substantially shifts the microbiome[633, 634]. MCAO is one of the most common animal models used in stroke research and forms the basis of the majority of this work. MCAO models have shown substantial shifts in the microbiome away from normal microbiome in the ceacum[633] and more broadly[634]. These differences have been identified at all levels of bacterial taxonomy from phylum to OTU[634], suggesting that post stroke microbiome analysis is flawed.

Aside from the neurological findings, the microbiome may contribute to stroke pathology through its effects on the vascular system in atherosclerosis[635-637] development, blood pressure[638, 639] and structural vascular lesions[640].

1.4 Microbiome and the Eye

The seeming isolation of the eye from the microbiome has limited the research effort assessing the potential for holobiont interactions in this organ. Even so, there is evidence that the microbiome has a role in ocular surface disease, inflammatory conditions, and retinal disease. Furthermore, there has been a small body of work that directly implicates microbiome in glaucoma.

The effects of dysbiosis in the tear film microbiome may be responsible for ocular surface disease. Ocular surface disease appears to be due to chronic inflammation of the cornea and conjunctiva. A mouse model of Sjogren's syndrome, involving the modification is of a gene involved in phagocytosis, causes both overgrowth of the ocular microbiome and subsequent conjunctival inflammation[641], suggesting a role in host-microbiome interactions leading to ocular surface disease. In dry eye disease models, specifically, it has been shown that elevation of the regulatory T cell response (which limits cellular inflammation) reduces disease[642, 643], which has raised the question of what is causing the inflammatory response in the first place[422]. There is some evidence from culture-based research that ocular surface disease may be associated with alterations in the makeup of the ocular microbiome[644], these findings, however, were not replicated in another similar culture based study[645].

It is clear that rheumatological conditions are an area of interest to microbiome researchers. Altered microbiome has been associated with rheumatoid arthritis[646], ankylosing spondylitis[647], and IBD[648] all of which are similarly associated with uveitis[649, 650]. HLA-B27, an antigen strongly associated with spondyloarthropathies and uveitic diseases, is an antigen that is expressed by a proportion of the population, with prevalence highly varying amongst different ethnic groups[651]. Interestingly, HLA-B27 is associated with a specific set of microbiome changes[652, 653]. The effects of HLA-B27 on the microbiome depend highly on the genetic background of an animal[654]. Due to these effects, the directionality of host-microbiome interactions is difficult to determine. However, there is evidence that the arthritic manifestations may be due to microbiome changes[652, 655, 656]. The microbiome also is a source of biomass that presents an antigen load that may contribute to molecular mimicry[657]. It has been noted, in a Chinese cohort, that alterations in the gut microbiome are associated with uveitis[658]. A number of groups have now shown that the microbiome may cause or worsen uveitis in animal models of uveitis[659, 660]. One group also showed that altering the microbiome with antibiotics can reduce the rates of uveitis through a T reg dependent mechanism[659]. This

area of research is beginning to expand, it is clear that research will progress to determine the extent of microbiome's role in uveitis.

With regards to retinal disease there have been several articles published; however, this area of work is still in its early infancy. There has been a single sequencing paper that has found differing microbiomes in macular degeneration patients when compared to healthy controls[661]. In this study, they noted both significant whole microbiome composition differences, specific changes at genus level, and also alterations of several metabolic pathways including alanine fermentation, arginine biosynthesis and glutamate degradation[661]. The potential association between the microbiome and diabetic retinopathy may seem more intuitive given the relationship between the former and diabetes, as a whole, however dissecting out the glucose independent effects remains difficult. One article demonstrated that intermittent fasting which has been shown to have a protective effect on the retina in diabetic retinopathy might mechanistically work through a microbiome mediated mechanism[662]. The potential for microbiome effects to be investigated in retinal illnesses is clear, however there remains much work before interactions will be relevant to clinical ophthalmology.

Finally, the microbiome's role in glaucoma, as investigated by other groups, is particularly relevant to this thesis. In addition to the recent work regarding microbiome mediated T cell driven autoimmunity by Chen *et al*[245], there is only limited research in this area. Of the published literature, there have been two arms of investigation undertaken so far. Firstly, there is some suggestion that the oral microbiome is involved in glaucoma. The first mention of this link in the literature appears to be an article published in 2014 that showed that glaucoma patients had higher oral bacterial counts and that intraperitoneal injection of LPS led to worse effects in experimental glaucoma[663]. The other area of potential microbiome glaucoma interaction explored in the literature is a body of work investigating if *H. pylori* infection could be related to Glaucoma, with a 2015 meta-analysis has showing a significant association[160]. *H. pylori* eradication was shown to be protective in glaucoma[158] (although this has been questioned in another study[161]), suggesting that antibiotic therapy for *H. pylori* may have effects in glaucoma. The antibiotics associated with *H. pylori* eradication have long-standing effects on the makeup of the microbiome[418]. Even if the effects of *H. pylori* do not indicate a broad microbiome effect in glaucoma, they do demonstrate the potential for gastrointestinal disturbances to effect glaucoma progression.

1.4.1 The Role of the Microbiome in Glaucoma Risk Factors

With regards to the central research question of this thesis, it is essential to address how microbiome relates to the currently known risk factors for glaucoma.

The risk factor for glaucoma with the most evident effects on microbiome is age. As already discussed the microbiome develops rapidly through childhood, achieves a relatively stable pattern in adulthood and then slowly changes as an adult moves into their senior years[394]. Given that, from an evolutionary perspective, the aging adult should have already produced progeny, it is not clear that holobiont homeostasis should be maintained in the individual of advanced age. Furthermore, human studies of aged microbiome (particularly centenarian microbiomes[411, 421]) may not display the typical microbiome changes or potential homeostatic breakdown in the elderly due to significant survivorship bias. Similarly, until causation studies are performed, it is not clear if the microbes associated with frailty seen on association studies are microbiome compensation or causative changes[664]. What is clear, however, is that the microbiome changes significantly as the human ages, and as aging is an important risk factor in glaucoma there is a potential interaction.

Similarly, although a weak risk factor for glaucoma, gender differences in microbiome have been noted[391]. If these differences are related simply to holobiont interactions whereby a different hormonal profile of the host will have different microbial inhabitants, or to what extent these differences provide functional alterations[415] is not known.

Heritability of the microbiome has also been discussed previously. It has been established that microbiome inheritance may be responsible for trait inheritance[455, 665, 666] and therefore it may play some role in the inheritance of illnesses. Proving the role of microbiome heritance in disease heritance will be difficult to prove in humans due the highly diverse microbial communities that an individual harbours[390, 391, 467] and will require vastly more complex studies than the already difficult association studies currently performed.

The microbiome and its role in diabetes has been the subject of great interest[466, 667, 668]. It is clear that people with diabetes contain interesting microbiome patterns[667, 668], some of which is due to the metabolically active medications diabetic patients use[466]. There is also some potential that microbiome alterations may predispose an individual to the development of diabetes[669]. Given the conflicting data regarding diabetes and glaucoma, the interactions between microbiome and diabetes should be considered more for their confounding role in microbiome glaucoma studies rather than as a causative pathway.

The diet is responsible for both superficial and profound changes in the microbiome structure[417, 461, 462]. Although the majority of glaucoma's diet related studies have focused on either caffeine or compounds that increase or reduce oxidative damage, it is likely that even these dietary associations may play a role in microbiome shift. Interestingly, one study demonstrated that the dietary intake of certain compounds was more closely related to glaucoma than the serum level of these compounds[670]; similarly two other studies showed that supplementation of certain compounds offered different effects than the dietary intake of these foods[137, 671]. It is conceivable that these findings point to a microbiome effect rather than a specific nutrient effect. It is possible that the consumption of vegetables high in these compounds offers protective microbiome changes that are not seen in supplementation and the consumption level does not necessarily translate into serum levels of these compounds.

Smoking appears to result in changes of the microbiome[672-674]. Smoking, like diabetes, is a controversial risk factor for glaucoma and therefore this relationship should similarly be understood for the purposes of confounding studies rather than as a causative link. Further work is required to determine how diet/microbiome effects may impact on host health.

OSA, in addition to wreaking havoc on the cardiovascular system, is likely to alter the microbiome through the effects of intermittent hypoxia and hypercapnia on the gut environment. In a mouse model of OSA, induced hypoxia and hypercapnia caused a marked alteration in the microbiome signature[675]. The authors of that study anticipate using this model to determine the role of the microbiome in OSA related comorbidities. Future work will clearly offer an intriguing look at the contributory effects of the microbiome and the direct effects hypoxia/hypercapnia in OSA related morbidity.

SES and rurality are both likely to impact on the microbiome profile of an individual. Within countries, there is accumulating data that shows a difference between rural and urban microbiomes in both the GIT[676-678] and other anatomic locations[679, 680], suggesting that lifestyle or SES factors may be significantly responsible for differences in microbiome. More than that, it is now well known that artificial sweeteners, preservatives, and food additives, which are more prevalent in the diets of lower SES status individuals[681-683], significantly alter the microbiome[684-687]. The western lifestyle has been blamed for the decreasing diversity of the microbiomes seen in the developed world[475, 476]. These factors also both increase the risk of certain environmental and occupational exposures[688, 689] which may also play a role in microbiome composition[690], although this is an area for future investigation. Fundamentally,

these factors both broadly describe lifestyle and from this an expected microbiome pattern may arise.

These findings are important to the planning of human microbiome-glaucoma studies as they help to identify potential confounding factors and also potential causative factors in the microbiome-glaucoma pathway.

1.5 Investigating the Microbiome in Epidemiological Research

Although both analysing the microbiome and identifying dysbiosis are difficult at present, current human microbiome research has focused mainly on cross-sectional studies that attempt to document differences in the microbiome between groups separated based on specific traits or illnesses. In most cases the group with the disease of interest are matched to a cohort of 'healthy' individuals and a post hoc analysis is performed. In many ways, the model for contemporary microbiome research is modelled on the GWAS studies that came before. This approach is flawed for several reasons. The microbiome, is substantially more complex than the genome, with higher high inter-individual variability, and a multi-kingdom mix of life that differs across anatomical regions[395]. But most importantly the temporal variation and its plasticity profoundly limit the conclusions that can be drawn from microbiome studies. As the microbiome acts as an interface between the host and the environment, cross-sectional studies are often not able to separate the effects a medication or treatments patients may be using from the illness of interest.

The majority of microbiome research addresses the bacterial component of the microbiome with little done to assess the other components of the microbiome: fungi, archaea, and viruses/bacteriophages. Even less has been done to evaluate the intra-microbiome interactions between the bacteria and the other organisms within the holobiont. Taking only the bacterial data into account leads to the potential for 'false negatives', whereby causative changes in non-bacterial microbiome may be missed, and 'irrelevant positives', where benign bacterial alterations, in response to microbiome members from other kingdoms, are flagged as pathologically relevant. Kingdom neutral analysis techniques are an aim of the microbiome field although challenging to achieve in practice. Shotgun metagenomics is suggested as the best potential option at the moment as all DNA is sequenced however sample preparation techniques may release DNA from some organisms more easily than others, and RNA based bacteriophages will be excluded merely through the restriction to assess samples at DNA level. Given the inter-kingdom assortment of life, contemporary sequencing techniques are deficient in capturing the whole picture of the microbiome.

The biogeographic specificities are will also play into the potential for dysbiosis to be identified. Given that each body region appears to have its own microbiome which appears

to be at least partially independent of other body regions[691], it is conceivable that dysbiosis may be completely missed by whichever sampling method is chosen.

Cross-sectional studies are unable to address the temporal aspect of the disease. Cross-sectional studies are unable to determine if the noted effects are persisting evidence of predisposing factors that contributed to the illness, a result of the illness with neutral effects, or a result of the with ongoing harmful or compensatory effects. Furthermore, a transient microbiome 'trigger' that may catalyse pathology could resolve at the level of the microbiome prior to the pathology being identified. For this reason, microbiome research must shift from small cross-sectional case-control studies to larger prospective studies so that the temporal relationship between microbiome disturbances and disease can be established.

Ideal studies addressing the role of dysbiosis in disease would identify abnormalities of the microbiome, over multiple time points. Until then, sequencing studies can only determine differences in the microbiome that are of unknown biological significance. Due to the cost and time-consuming nature of such studies, the identification of a marker of disturbed microbiome, particularly one that is relatively easy to elicit in population-based studies, would allow for more rapid testing of hypotheses, particularly in established longitudinal data-sets that may already have these data.

1.5.1 A Pathomarker for Dysbiosis

The idea of the pathomarkers was conceived to identify dysbiosis in studies that have not explicitly assessed for it. We define a pathomarker as a pathology that is strongly correlated to an exposure measure and can, therefore, be used as a 'red flag' for identification of the exposure measure in population-based research. The authors propose that pathomarkers should be of interest to epidemiologists when addressing outcomes measures that are currently difficult to define, identify or measure, in population studies, such as 'dysbiosis'. Using a pathomarker in preliminary studies can inform the design of large prospective studies to determine the burden of dysbiosis in illnesses with low incidence or that are unlikely to be analysed in initial prospective studies.

The use of a pathomarker should allow for lower cost, quick analyses of concepts. In many cases, if the pathomarker is a moderately common disease process, data may already be available in large publicly available population-based studies. When data has already been collected in large studies, testing associations are especially cheap. Pathomarker association

studies, especially for physiological states such as dysbiosis, may be employed as pilot studies to guide further research into the underlying exposure measure.

A useful pathomarker for identifying a poorly characterizable exposure variable, such as dysbiosis, needs to meet several criteria:

Criterion 1: The pathomarker must easier to identify that the exposure it marks. Although this consideration is logical, it should be articulated. There is no utility in a pathomarker, if its identification is more difficult than the exposure measure that it marks.

Criterion 2: The pathomarker must be 'specific' for the exposure it marks. It is likely that the underlying exposure for which the pathomarker is employed will be relatively uncommon in the population being assessed. Pathomarkers should work best when the majority of participants with the pathology have been exposed to the underlying exposure it marks. Theoretical statistical modelling has demonstrated that when exposure rates are approximately 10% (or lower), the specificity of the exposure measure is more important, than the sensitivity, for minimising the magnitude of bias[692]. The same modelling suggested that sensitivity is only clearly more important than specificity once the exposure rates reach approximately 50%[692].

Criterion 3: The pathomarker must be reasonably limited in its pathological breadth so that confounding factors can be identified and assessed. Along the same lines as the above regarding specificity, a significant and wide-spread pathology is less usable as a pathomarker as the physiology of the illness may impact on pathways that are irrelevant to the underlying exposure. Similarly, the symptomatology of the pathomarker may mask or accentuate the outcome assessed. The limits of a pathomarker should be identified and noted in analyses where symptomatology or pathological mechanisms may cross over, and caution should be used interpreting results that may be significantly affected by this issue. Furthermore, if any aspect of the pathomarkers symptomatology could (outside of the physiological effect of the underlying exposure) impact on the identification of the outcome measures, bias away from the null may result[693, 694] and therefore the pathomarker should not be used.

A number of factors that guide the utility of a pathomarker are nevertheless irrelevant to the validity of the pathomarker.

The sensitivity of the pathomarker to identify the associated poorly identified exposure is irrelevant to its validity. A pathomarker with poor sensitivity (i.e. a pathology that only marks a small proportion of the exposed participants) remains valid since non-differential

misclassification bias theoretically biases towards the null. Mathematically, the exception to this rule is when the sensitivity and specificity of the marker are so low that when added together are less than 1[695].

The association between the exposure and the pathomarker is not necessarily due to a known or specific causal direction between the two. By definition a pathomarker is *associated* with the poorly measured exposure it represents. Given that the pathomarker should be specific for identifying the unmeasured exposure it represents, there is a high likelihood that the there is some causal link between the two. It is not incumbent upon the researchers employing the use of the pathomarker to identify the pathophysiology linking the pathomarker to the poorly defined outcome, it is, however, important that the researchers address that the underlying outcome may play a role in the hypothesized outcomes via a pathway dependent or independent of the pathomarker, or the pathomarker may cause the hypothesized outcome by causing the underlying poorly described outcome (Figure 1.3).

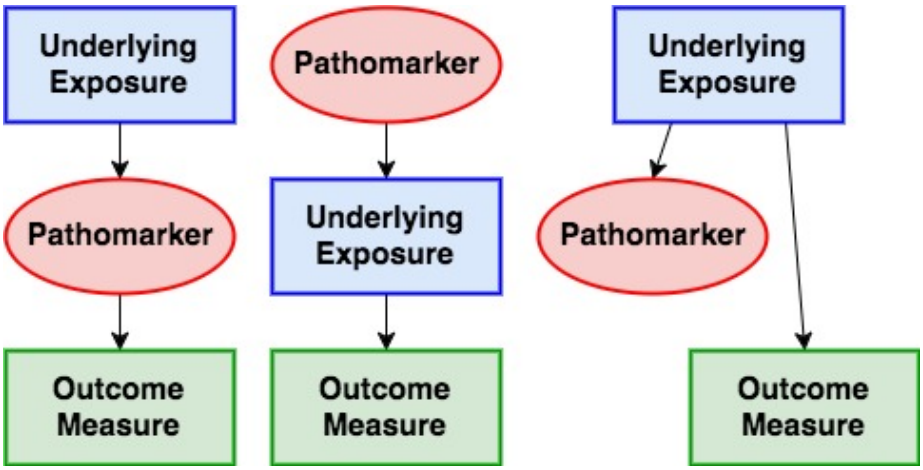


Figure 1.3: Potential causal pathways that explain the real association between a pathomarker and an outcome measure with relation to the underlying exposure

1.5.1.1 Irritable Bowel Syndrome

IBS is a common gastrointestinal illness with a relatively clear clinical picture. IBS is often defined as abdominal pain that is at least partially relieved by defecation. It is considered as a functional disorder as research has yet to define any structural or metabolic abnormalities that are sufficiently robust to explain symptomatology. Its pathogenesis is multifactorial and likely includes a spectrum of contributing factors including visceral hypersensitivity, psychological factors, dysmotility, low-grade inflammation and alterations

in the brain-gut axis, in addition to genetic and environmental factors. The microbiome has been suggested as an independent modifier of IBS pathology and may be a super-regulator of the above mechanisms.

IBS subtyping typically occurs with the distinction of patients based on the form of their faeces. Within the illness there are four generally recognised subtypes; Diarrhea prominent IBS (IBS-D), Constipation prominent IBS (IBS-C), Mixed type IBS with both diarrhea and constipation (IBS-M), and Post-Infectious IBS (PI-IBS). IBS-D and IBS-C are indicated in patients who pass >25% of their stools as loose or watery stools or had solid lumps, respectively. IBS-M is defined by meeting the criteria for both IBS-D and IBS-C[696]. PI-IBS, whilst still considered a sub-type of IBS, is a diagnosis based on IBS symptoms being initiated shortly after an episode of gastroenteritis. The majority of patients with PI-IBS meet the criteria for IBS-D[697]. The question of whether IBS subtypes are representative of distinct illnesses is a question that has been debated without consensus.

With a heritability estimate of 27-57%[698] the majority of IBS variance in the community likely occurs due to non-genetic reasons; environmental factors are considered a highly important etiological factor.

IBS is Readily Identified

IBS is a relatively common illness with a prevalence estimated to be 11.2% worldwide[699]. Its prevalence is known to vary regionally from around 7% in South Asia to approximately 21% in South America[699]. IBS is approximately 1.67 times more prevalent in females than males[699] but this discrepancy also varies in different regions. IBS occurs in all age groups from childhood into the elderly with little variation in subtype distribution across different age groups, although abdominal pain symptoms tend to be milder in older patients[700]. Although there is no clear relationship between age and IBS, more than half of patients report having their first IBS symptoms before the age of 35[701].

Currently, the definition of IBS revolves around the identification of a constellation of symptoms in addition to the absence of an alternate organic diagnosis. The vast majority of gastroenterologists use the definition of "Abdominal pain with disordered defecation"[702], however, more detailed diagnostic protocols are available including the ROME criteria[703] and the Manning criteria[704]. Although these different methods can define IBS, there is a high

degree of agreement between these for the identification of IBS, allowing for a significant amount of external validity of research involving IBS pathology[705].

Misdiagnosis appears to be a minimal issue for IBS. People who had received a diagnosis of IBS were no more likely to have organic lesions (which would indicate misdiagnosis) found on colonoscopy than a matched cohort of healthy controls[706]. That said, IBS is considered as a risk factor for Inflammatory Bowel Disease[707]. Some have argued that this indicates that IBS symptoms may be, in some, early signs of Inflammatory Bowel Disease, however, others have argued that this may indicate that there are some pathophysiological mechanisms shared by these two pathologies, such as microbiome disturbances[708, 709].

In contrast to gastrointestinal dysbiosis, IBS is significantly simpler to identify and can be assessed with multiple sensitivity levels (physician diagnoses, patient self-report or through utilisation of diagnostic questionnaires). As such, IBS meets the first criteria for a pathomarker of dysbiosis.

Association between IBS and Microbiome Disturbances

The association between a disturbed gastrointestinal microbiome and Irritable Bowel Syndrome is well accepted by gastroenterologists and microbiome researchers[710, 711]. As has already been discussed, establishing causality in human microbiome research is difficult with current study designs.

Microbiome Disturbance May Precede IBS Pathology

It is clear that a large proportion of IBS is preceded by a significant disruption in the gastrointestinal microbiome. An acute bacterial or parasitic infection of the Gastrointestinal system is the strongest predictor of IBS incidence[712]. The link between the use of antibiotics and the incidence of IBS is less established. In appropriately controlled prospective studies, antibiotics appear to be associated with increased levels of functional gastrointestinal symptoms[713, 714] and moderately associated with incident IBS specifically[714]. The effects of antibiotics may also explain the unexpected finding by McKeown *et al.*, that non-GIT infections were associated with a 6 fold increase in IBS at 3 months after infection[715].

IBS Pathology is Universally Associated with Bacterial Dysbiosis of the Gastrointestinal System

There is a rapidly growing consensus in the literature demonstrating that Irritable Bowel Syndrome is associated with a gastrointestinal microbiome that is not typical of 'healthy' controls.

There have been several case-control studies demonstrating that the microbiota of IBS patients is compositionally different from healthy controls. Case-control studies have consistently demonstrated that the decreased diversity[716-720] and altered richness[721-723] of the microbiota in IBS patients. These diversity/richness associations are limited in their capacity to identify 'dysbiosis' however they do indicate that people with IBS have a significantly different taxonomical make-up of their microbiome compared to healthy controls. Recent systematic review found no clear trends at the phylum level[724]. When analysed at the genera and species levels, some consistent results are seen[724]. The *Ruminococcus* genus and certain of its species have been shown to be elevated in people with IBS with a fair amount of consistency[710, 725-729]. *Bifidobacteria,* a genus of 'beneficial' bacteria, has been found to be decreased in IBS patients with consistency[724, 726, 727, 730-732]. There is less consistency in with regards to the *Lactobacillus* genera, which have been demonstrated to be elevated in some studies and reduced in others[710]. In meta-analysis, though, both *Bifidobacteria* and *Lactobacillus*, as well as *Faecalibacterium prausnitzii*, were seen to be reduced in IBS patients[733].

The differences in the faecal microbiome are different from the differences demonstrated by the mucosal microbiome. The colonic mucosa seems to present smaller numbers of species and genera specific differences in the microbiome compared to faecal samples when comparing IBS to healthy controls[734, 735]. That said, the rectal mucosal microbiome demonstrated distinct microbiomes and was able to differentiate IBS from healthy controls and IBS subtypes from each other[736]. Whilst IBS, in general, contains a microbiome that is distinct from healthy controls, the various subtypes of IBS also show differing microbial signatures allowing distinctions within IBS subtypes[726, 737, 738]. Although less well described, there is also evidence that there are functional changes in the microbiome[716, 739], and the metabolites the microbiome produces[740-743], in IBS patients.

Taking the next step, three studies have demonstrated the utility of profiling the faecal microbiome in the diagnosis of IBS[721, 741, 744], one of which accurately sorted people suffering IBS into various symptom severity levels[721].

The symptoms of IBS themselves may also be explained by alteration to the functions of the microbiome. IBS patients with bloating have a microbiome signature that is distinct from IBS patients without bloating symptoms[745]. In agreement with this, altered colonic fermentation has been described in IBS patients[746, 747]. Moreover, patients with IBS, particularly IBS-D and IBS-M, have also been shown to have reductions in methane-producing bacteria, which can lead to hydrogen accumulation potentially explaining bloating symptoms[716]. Microbiome regulation of gastrointestinal motility was implicated by the recent association of the microbiome profile to constipation and transit time in IBS patients[748]. In humanised mice, colonic transit time was associated with microbiome changes and had complex interactions with diet[749]. In another humanised murine model, the microbiome from IBS patients elicited colonic hypersensitivity in humanised GF rats[750]. Similarly, antibiotic-induced microbiome alteration is also able to cause colonic hypersensitivity in mice[751]. In human studies, several taxa have also been linked to abdominal pain symptomatology[716]. Taken together these results demonstrate that dysbiosis of the microbiome can be a significant cause of the features seen in IBS pathology.

Beyond the colon and the faeces, there is also growing evidence that in IBS patients the microbiome is different, to healthy controls, in the jejunum[752], duodenum[753, 754] and even the oral cavity[722]. A recent analysis of the microbiome from the Jejunum, collected by tissue sampling at endoscopy, demonstrated that people with IBS have reduced levels of oral flora compared to healthy controls and that the levels of Neisseriaceae were inversely associated with IBS severity[752]. Alterations in the composition of the microbiome of duodenal mucosa have also been described[753, 754], with specific findings demonstrating a decreased abundance of bacteria from the *Bifidobacterium* genus[754].

Temporal stability of the microbiome, an indicator of the homeostatic function in host-microbiome symbiosis[396, 755], has been found to be reduced in three cohorts of IBS patients as compared to healthy controls[756-758]. This temporal instability may also explain why inconsistent and occasionally conflicting data are presented in cross-sectional studies of the taxonomy of the microbiome where only one sample is taken per participant.

Distinctions can also be made within the microbiomes of IBS subtypes[726, 737, 738]. Whether the various subtypes of IBS represent separate pathologies is debated[696]. Given that all the IBS subtypes demonstrate microbiome abnormalities, analysing the pooled group of IBS as well as IBS subtypes when using IBS as a dysbiosis marker adds robustness to analyses.

Similarly, subgroup analyses may indicate that the dysbiosis associated with a specific subtype is more relevant to the outcome of interest.

There is significantly less understanding of the non-bacterial component of the microbiome. A recent study demonstrated that the virome is expanded and more diverse in inflammatory bowel disease compared to healthy controls, and these findings were not secondary to changes in bacterial populations[759]. At present, it is unknown if the virome is altered, in a similar manner, in people with IBS. The fungal component has been assessed recently in one large study. In this work, Botschuijver *et al*. found that IBS patients exhibited a fungal microbiome that differed significantly from healthy individuals compositionally in both richness and evenness[760]. The predominant fungal species *Saccharomyces cerevisiae* and *Candida albicans* were significantly more abundant in IBS patients compared to healthy controls[760]. Other differentially expressed species had less clear distributions[760]. Even so, it was evident in this data that the fungal microbiome differed between IBS patients and healthy controls[760].

At present, the data supports the use of IBS as a marker of bacterial dysbiosis; however more research is required to understand the non-bacterial component of the microbiome in IBS.

Altering the Microbiome May Resolve IBS Symptoms

There are a number of interventions that have been suggested for the treatment of IBS. Many of the suggested therapies for IBS have direct effects on the composition and functions of the microbiome.

Rifaximin, a poorly absorbed gut-specific antibiotic, is the best-studied antibiotic for the treatment of IBS. A meta-analysis of five randomised placebo-controlled trials demonstrated that rifaximin was more efficacious than placebo for global IBS symptom improvement and improvement in bloating, pain and stool symptoms[761]. In a study of humans taking rifaximin for IBS treatment, microbiome from stool samples demonstrated a shift in the microbiome toward a picture similar to healthy controls although with incomplete resolution[762].

Probiotics have been demonstrated to have benefit in IBS, with a demonstrable resolution of symptoms[763]. The primary hypothesis regarding their mechanism is that

probiotics alter the microbiome, shifting it toward a healthier homeostatic point, although the efficacy of probiotics to achieve this is questioned[718, 764-769].

The low-FODMAP diet is another treatment option for IBS sufferers. Fermentable Oligo-, Di-, Mono-saccharides And Polyols (FODMAPs) are a group of short-chain carbohydrates that are poorly absorbed in the small intestine[770]; it has been suggested that the abnormal fermentation of these nutrients by a disturbed microbiome is responsible for bloating and other symptoms of IBS pathology[770]. Two recent systematic reviews have concluded that reduced FODMAP diets are efficacious for the treatment of IBS in the short term[771, 772]. Importantly, as well as having effects on the fermenting function of the microbiome, a diet low in FODMAPs has marked effects on the composition of the microbiome[773]. In one particular randomised control trial, the microbial composition of the people who responded best to a low FODMAP diet had specific alterations in their microbiome with particular abnormalities around carbohydrate metabolism[774].

Faecal Matter Transplant (FMT) has been suggested, albeit with limited scientific validation, as an IBS treatment. At present, there are two published RCT's on the use of FMT for IBS[775, 776], with conflicting conclusions. More research is required in this field before FMT can be adopted as a therapeutic option.

These findings also meet several of the Bradford Hill criteria[777] in the relationship between dysbiosis and IBS (Table 1.1). The demonstration of dysbiosis preceding IBS, and microbiome modulation being used as a therapy, are strong evidence of 'Temporality'. All these data suggest 'Consistency' in the association between IBS and dysbiosis. Finally, the fact that a number of articles have suggested that the composition of microbiome may be used diagnostically suggests a great deal of 'Specificity'.

Table 1.1: Summary of the associations between microbiome abnormalities and IBS, classified according to the Bradford Hill criteria

CRITERIA	EVIDENCE
STRENGTH	• Intestinal infection is associated with odds ratio of 7.58 for developing IBS in the next 3 months[712].
CONSISTENCY	• Case-control studies have consistently demonstrated decreased diversity[716-720] and altered richness[721-723] in IBS patients. • Members of the *Ruminococcus* genus are more abundant in people with IBS[710, 725-729] • Meta-analysis demonstrated people with IBS have reduced abundance of *Lactobacillus*, *Bifidobacterium* and *Faecalibacterium prausnitzii*[733]. • A recent systematic review found that Enterobacteriaceae, and *Bacteroides* were increased in patients with IBS, whereas uncultured Clostridiales I, *Faecalibacterium* (including *F. prausnitzii*), and *Bifidobacterium* were decreased[724].
SPECIFICITY	• Microbiome differences have been used to predict IBS diagnosis based solely on microbiome profile with up to sensitivity of 81.8 %, and specificity 86.4%[741]. • Microbiome profile was able to predict symptom severity with 82.9% sensitivity with 80% specificity[721]
TEMPORALITY	• Clinical microbiome disturbance (determined by antibiotic usage, and GIT infection) precede IBS in a significant proportion of cases.
PLAUSIBILITY	• Bacteria in the GIT are responsible for digestion of food and may produce gas through fermentation processes leading to bloating, specific bacteria have been linked to bloating symptoms[716, 745]. • Microbiome profile has been associated with colonic transit time in animals[749] and humans[748]. • Several bacteria have been linked to abdominal pain symptomatology[716].
COHERENCE	• The microbiome from IBS patients elicited colonic hypersensitivity in Germ Free rats colonized with human microbiome[750].
EXPERIMENT	• low FODMAP diets are efficacious for the treatment of IBS[771, 772]. • The antibiotic rifaximin, which shifts the microbiome toward normal[762], is efficacious for IBS symptom improvement[761]. • Some probiotic therapies have demonstrated symptomatic benefit in IBS patients.

The Breadth of IBS's Physiology is Limited.

If IBS is to be considered as an indicator for dysbiosis, systemic pathology as a component of IBS pathology must be understood and accounted for. There is currently no specific biomarker or panel of biomarkers that can accurately identify IBS from the healthy population[778] which suggests that there is a reasonably limited organic pathology outside of the established symptomatology. Currently, only two 'biomarker panel' studies have achieved moderately successful results. A panel of 10 biomarkers proposed by Lembo *et al.*[779] demonstrated Area Under the Curve (AUC) of 0.76 for discriminating IBS from healthy individuals. A separate panel of 34 serological biomarkers proposed by Jones *et al.*[780] demonstrated AUC of 0.81, and AUC of 0.93 when 4 psychological markers were included.

Despite being considered a functional condition, gastroenterologists have, for years, suspected that IBS is a low-grade inflammatory disease[781-783]. Low-grade inflammation of the intestinal mucosa has been described in the Post Infectious subtype of IBS[784, 785]. It is suspected that in PI-IBS, patients are unable to down-regulate the inflammation in the colonic mucosa that was associated with the gastroenteritis[783]. In one particular study, peripheral blood mononuclear cells (PBMCs) were isolated from people with IBS and healthy controls. These cells were cultured with LPS for 24 hours to assess the immune reactivity of these cells. Basal levels of the cytokines tested (TNF-α, IL-1β, and IL-6) were elevated in PBMCs from IBS patients at baseline; although when grouped by subtype of IBS, IBS-D accounted for most of the deviation from the mean[786]. These results are consistent with previously reported mild elevations of IL-6 and IL-8 levels in IBS patients[787]. In a more recent study, however, these results were not validated in a cohort of IBS patients without PI-IBS[788].

T cells have been of interest to IBS researchers regarding their relationship to inflammation in the GIT. Although regulatory T cell numbers are comparable between IBS patients and healthy controls[786, 789, 790], IBS patients' T cells express the 'gut homing' integrin-β7 at much higher rates[791, 792]. Whether these T cells are responsible for local inflammation in the gut, or they represent a marker of gastrointestinal inflammation, is not definitively proven at this stage[793].

Is it possible that these immune system effects are due to microbiome alterations? Serum levels of IL-6 can be altered in humans in response to alterations in the microbiome and diet[794]. Animal studies have also shown that perturbation of the microbiome alters the production of IL-1β[795, 796], and IL-6[796]. An *in vitro* study of human monocytes, also,

demonstrated that specific bacteria can modulate TNF-α[797]. TLR receptor function is another immunologic domain that has been shown, in multiple studies[798-800], to be dysregulated in IBS. Animal studies have been used to show that the microbiome significantly alters the functions of TLR's[751, 801] and that TLR dysfunction creates dysbiosis[802, 803].

Taken together these results suggest that the microbiome can affect the immune system, specifically in ways that are associated with IBS. It follows, however, that if IBS is to be considered as a marker for dysbiosis, care must be taken to evaluate the potential for low-grade inflammation to be causing any effects seen.

The psychiatric comorbidity associated with IBS is well known[804, 805]. A hyper-reactive response of the HPA axis has been described in IBS patients potentially linking stress to symptomatology[787, 806, 807]. Moreover subclinical psychological stress[808, 809] and adverse events early in life[810, 811] can precede IBS. Psychiatric disorders and IBS appear to have bidirectional effects on each other[812, 813]. It is of interest, therefore, that the microbiomes effects on the HPA axis closely parallel the bidirectional effects seen between IBS and psychiatric illness. It has been long known that stress can have lasting effects on the microbiome as described in human[814], primate[815] and murine studies[816-819]. Sudo *et al*. first reported that the microbiome-HPA axis effects were bidirectional in 2004, demonstrating that the corticosterone and adrenocorticotropic hormone response was greatly elevated in GF mice[513]. These results have been confirmed numerous times and have also been correlated to behavioural abnormalities typically associated with depression and anxiety[514, 515, 820]. Interestingly, a human study also demonstrated a role for probiotics to impact human neural physiology as described by functional MRI studies[821]. As with inflammation, it is possible that the microbiome plays a significant role in the association between IBS and psychiatric illness; however, care must be taken in the interpretation of results linking IBS associated dysbiosis to illnesses that may also be associated with psychiatric outcomes.

Shared Risk Factors for IBS and Glaucoma.

Using IBS as a pathomarker for dysbiosis in an analysis for the potential role for the microbiome in glaucoma requires an understanding of their shared pathology and risk factors. To our knowledge there are no ocular risk factors for IBS; however, a number of the other risk factors for glaucoma are worth discussing regarding their potential to confound any association.

Demographic factors are important in any association study. Age is a very strong risk factor for glaucoma with a steep incline in prevalence after age 40. The association between age and IBS is less clear. The illness may occur in any age group and has been described in the elderly and children[706]. Although it is considered a lifelong illness, the prevalence over the age of 50 is 75% the rate of the prevalence seen in those under the age 50[699] suggesting that symptoms may resolve with age. In meta-analysis there is a female gender association with IBS[822] however this is not clear in all populations. Similarly, in glaucoma, the relationship to gender is not clear. In unadjusted cohorts[77], glaucoma is often more prevalent in women than men simply due to the difference in life expectancies. However, when adjusted for age, there appears to be a male predominance[25]. It is important that the demographic factors of age and gender are considered in research assessing IBS and glaucoma.

The effect of genetics and ethnicity is well appreciated in glaucoma. African heritage is a strong risk factor for glaucoma in meta-analysis, with Asian and Hispanic decent offering insignificant elevation of risk compared to white populations[17]. Those of Asian descent are, however, much more likely than Caucasian populations to have angle closure glaucoma[17]. Ethnicity's effects are less clear for IBS; international systematic reviews of IBS epidemiology have found widely varying estimates varying widely by nation[706]. Another systematic review of prevalence rates was unable to find a significant difference between the western and Asian populations, although there was a trend that higher prevalence was seen in western countries[823]. In a Scottish study, Pakistani women were found to have lower rates of IBS although Pakistani men had similar rates to white men[824]. Given the unclear results in the IBS literature, it is not known how ethnicity will impact on the association between IBS and glaucoma.

Diabetes mellitus is growing to be one of the most significant lifestyle factors in modern medicine due to the effects of hyperglycaemia on the human body. The role of the microbiome in Diabetes is being explored with great vigor[667, 825, 826]. Diabetes has been associated with increased rate of gastrointestinal symptoms[827]; however the association between IBS and Diabetes seems minimal[828]. Although IBS prevalence in a Diabetic population was in the higher range typically seen, at 27% (with no nondiabetic comparison group), this was unrelated to glycaemic control[829]. The role of diabetes in glaucoma is similarly murky with meta-analyses demonstrating small positive associations[109-111]. Although the relationship between both IBS and glaucoma is weak at best, this is one area where confounding could

conceivably bias away from the null. For this reason, it is important to correct for diabetes in analyses.

OSA is increasingly recognised to be well associated with Glaucoma. This association has been suggested to be due to the transient hypoxia that the retina is exposed to, Given that the gut is already a relatively hypoxic environment[830], there is a potential for OSA to further increase the normal gut hypoxia which could cause microbiome shift[675] or may conceivably exacerbate gut symptoms associated with IBS. Indeed, the rate of IBS is significantly higher in people with OSA, suggesting that this could confound the relationship[831].

Smoking might be a risk factor for glaucoma with some studies demonstrating a positive relationship between smoking and glaucoma[115, 120]. A 2017 systematic review was unable to confirm a significant association between IBS and smoking, however of 33 articles assessing smoking prevalence between IBS and controls, 7 showed a positive correlation and 1 showed a negative correlation[832]. If people with IBS are more likely to smoke and this could cause glaucoma, this becomes an indirect causal path that should be considered for its relationship to the association.

The issue of SES is challenging to address because it is assessed by so many different methods in so many different contexts. There may be an association between urbanisation and glaucoma[17, 833, 834], however, the effects are inconsistent and have not been held up by other groups[169, 170]. Similarly, the relationship between SES and IBS is also unclear and depends on definitions used. When assessing the same urbanisation effects, it appears that IBS may be seen more in urban populations[706, 835] although other hypotheses may better account for these effects, including the greater healthcare accessibility, implying increased medical surveillance, in urban areas[836]. The heterogeneity of these studies makes designing valid studies that account for the SES of their populations difficult.

Given that the relationship between infection and IBS has been well investigated, it is quite clear now that *H Pylori* is not associated with IBS[837-839], and therefore does not confound this relationship.

1.5.1.2 Oral Health

Oral health may also be an effective pathomarker for dysbiosis, particularly of the oral microbiome. As has been described before, the oral microbiome is substantially different to the microbiome found elsewhere in the holobiont, although its community structure is

roughly as complex as the gut microbiome[391]. There are a number of different habitats within the mouth, from mucosa to bone, that offer distinct environments for various different microbes[840]. Dysbiosis may be related to dental disease, and again like IBS the directionality of this is not always clear, however it's possible that dental health may also offer a pathomarker for dysbiosis research. Prior to beginning this research task, there had already been research published to suggest that the oral microbiome may be related to glaucoma, described in this thesis elsewhere.

Even more so than IBS, dental health can often be described in discrete binary events (i.e. the loss of a tooth) that makes identification of these factors very feasible in population studies. Indeed, the oral health can be assessed by the number of teeth, incidental loss of teeth, as well as other more specific oral diagnoses. Amongst the more specific diagnoses such as periodontitis and caries, these are both significant causes of tooth loss[841, 842], and therefore population based survey studies that ask questions about tooth loss, even if dental illnesses are not diagnosed, may offer an opportunity to assess the oral microbiome at a very coarse but potentially compelling way.

Caries, also known as cavities, result from a multifactorial bidirectional interaction between the microbiome and the teeth. The teeth are the only body surface that does not shed making it a unique habitat for biofilm creation[840]. It is suggested that the risk factors that contribute to caries formation (i.e. sugar intake and soft drink intake) lead to a more acidic environment in the mouth shifting the microbiome toward bacteria suited to this environment, and eventually perhaps to acid producing bacteria which further potentiate this situation[840]. *Streptococcus mutans*, and anaerobic microbes appear to be related to caries[843, 844], and more broadly oral microbiomes of people with caries are significantly different to the healthy people[845-847]. Interestingly, in studies of the temporality of the relationship between bacterial disruption and caries formation, the microbiome shift occurs early, and perhaps even before the onset of illness[847, 848]. Research is ongoing to determine if microbiome modification, particularly through antibiotics targeting caries generating bacteria, can alter the course of this illness[843, 849].

Periodontitis is defined by destructive inflammation of the gums and supporting structures of the teeth. Accumulation of biofilm triggers gingivitis, the precursor of periodontitis[850]. Complex interactions between immune response mediators and biofilm are necessary requirements that lead to disease progression from gingivitis to periodontitis[840].

Dysbiosis of the microbiome is suggested to drive inflammation which creates a feedback loop favouring the growth of abnormal microbes, such as anaerobes and protein dependant bacteria[851]. This dysbiosis may then lead to further inflammation and micro-ulceration of the epithelium, which allows blood and iron to enter the gingival crevice which may cause the flourishing of periodontitis associated microbes[852]. The microbiome of people with Periodontitis is clearly different from the healthy controls, and has been noted in a number of different locations in the mouth[845, 853]. Finally, chlorhexidine (antiseptic) mouthwash is a legitimate therapeutic option for gingivitis, suggesting a reversible component to the microbiome/gum health association[854]. Combined, the dysbiosis that occurs in the oral microbiome in periodontitis, and the resultant inflammation, both may contribute to systemic illness, and therefore periodontitis may be a marker of oral dysbiosis suitable for epidemiological analysis.

Tooth loss may also be the outcome of oral dysbiosis. Periodontal disease is a major cause of tooth loss in adults in the developed world, responsible for half of tooth loss in one study of American adults[841]. Similarly caries are also a major cause of tooth extraction[855]. Although different studies tend to find caries or periodontitis to be the primary cause of teeth extractions depending on the population looked at, combined they tend to be responsible for up to 90% of tooth extractions[856-858]. The problem with the total number of teeth, and tooth extractions, in their use as a pathomarker for dysbiosis is that, although a dysbiosis related illness (i.e. caries and periodontitis) appears to be associated with most teeth extracted, they may also be lost due to injury, amongst other causes, suggesting that missing teeth do not necessarily indicate underlying dysbiosis. On the other hand, though, a lost tooth changes the habitat of the mouth, and may cause inflammatory events that could result in dysbiosis[852]. Furthermore, tooth loss is a clear break in the normal barrier between the host and the microbiome. Exposure of the circulation to the microbiome is a clear breakdown in normal homeostatic mechanisms, and this often leads to bacteraemia[859, 860]. For these reasons, it's clear that tooth loss is a dysbiosis event, even in the cases where dysbiosis was not causative in the tooth loss itself.

The primary issue with oral health as a marker for dysbiosis in epidemiological research is the fact that dental health is also associated with a significant number of risk factors that may confound associations. Oral health is highly associated to ethnicity[861-863], age[862, 863], gender[862, 863], dietary patterns[864-866], diabetes[867-869], smoking[870, 871], cardiovascular

illness[872-874], alcohol intake[875-877], and caffeine intake[878-880]. Many of these are significant risk factors for glaucoma (as has been discussed previously), and therefore association maps should be considered to identify confounders and indirect association patterns in associations to be considered.

1.5.2 Identifying Microbiome Effects in Animal Studies

Dysbiosis is a difficult concept to identify in heterogeneous populations of humans however many techniques can be used in animals to assess the role of the microbiome in health. The models used most frequently are GF mice, antibiotic treatment models, infection models, and microbiome transfer models. Each of these models have been discussed to some extent in previous chapters, particularly with regards to the experimental efforts to link the microbiome to phenotypes.

Germ Free Animals

GF animals have been used to assess the effects of the microbiome on animal health since the development of reliable GF equipment and techniques in the mid 20th century[881, 882]. Modern GF isolators are made of transparent flexible plastic allowing for a good field of view, and include many quality of life features to help assist researchers and animal care technicians perform experiments whilst maintaining sterility[883]. These isolators are difficult to maintain and laboratories using them require a staff of trained technicians to maintain them[883]. All bedding, food, water, and other equipment must be sterilised (usually autoclaved, or sprayed with germicidal vapour) and are then brought into the isolator through an airlock[883]. GF colonies are established by delivering mouse pups by a sterile caesarean section and then transferred whilst still in the uterine sac to a GF foster mother[884]. These mice are then allowed to breed, and the subsequent generations may be used for experimental work[884]. GF status is monitored regularly by culturing faecal samples for aerobic and anaerobic bacteria and fungi and by assessing 16S DNA on PCR[883]. A contamination requires total decontamination of the isolator and euthanising the residential colonies, as these animals are no longer usable for GF experiments.

The nutritional requirements of GF mice may be slightly different to SPF mice as the lack of microbes indicates that microbiome derived nutrients will be unavailable to these mice

and must be supplemented if essential for life. The most important nutritional supplement requirements for GF mice are vitamin K, without which animals may die from haemorrhagic diathesis[885], and vitamin B12, for which deficiency may cause growth restriction and renal impairment[886]. Assuming that the GF diet is supplemented with these vitamins, they remain healthy and live normal lifespans[883]. These vitamins are generally needed by SPF mice, as well, and are fairly standard supplements in standard lab diets[886].

There are a number of clear differences in the anatomy and physiology of these animals which must be considered in experimental work. The biggest anatomical difference is the huge difference in the size of the caecum – which may be 4-8 fold larger than in GF rodents due to the accumulation of mucus and undigested fibers[887]. Aside from this, most anatomical differences between GF and SPF mice are more subtle and include decreased body fat percentage, differing intestinal morphology, and decreased liver size[883]. Al-asmakh *et al*. have summarised the identified anatomical and physiological (metabolic and endocrine) differences between GF and SPF mice that have been identified[883]. The baseline differences in GF and SPF mice should be considered when assessing the results of experimental models in GF mice where anatomical differences may cause a seemingly altered behavioural pattern that may be simply explained.

By providing a binary, all or nothing, approach to the normal microbiome, these models are highly effective for answering the question of whether the microbiome could plausibly be related to the phenotype of interest. If the microbiome plays a positive effect or exacerbates a pathway involved in pathology, both circumstances will be identified in GF animals. Perhaps the only microbiome mediated effect that a GF model may miss is a gain of function effect caused by the infection of a healthy animal with additional non-typical microbes. In these circumstances, if the microbiome plays no role in a pathway, except with the addition of atypical microbes, then the pathways microbiome sensitivity will not be seen by comparing GF to SPF models. However, one may argue that those circumstances are less relevant baseline health and more relevant to therapy discovery.

The primary downside of GF research is the cost of the development of the models. As stated, the animals must be maintained in isolators that are maintained by a staff of trained individuals, above and beyond the typical training required for normal SPF animal husbandry. The derivation of a GF strain is difficult and requires precision and care to prevent contamination[881, 884]. Even when all standard precautions are taken, contamination of an

isolator may still occur[888], as various equipment and consumables will be introduced to the isolator over the course of life of a generation of mice. Contamination can be devastating for research with long follow-up periods as mice become unusable once contaminated. GF research, therefore, requires constant logistical manoeuvring to prevent contamination, to minimise the damage of any potential contaminations and to minimise the costs of underutilised isolators.

Antibiotic Models

Antibiotic models take two broad forms, antibiotic depletion and antibiotic dysbiosis models. Antibiotic depletion models involve the use of high dose broad spectrum antibiotic cocktails to eliminate a significant amount of the microbiome. These models are designed to mimic GF models and are much cheaper than establishing GF colonies. Antibiotic dysbiosis models use much smaller doses of antibiotics (often monotherapy) to cause a shift in the microbiome without (intentionally) depleting the total amount of microbes in the animal. These models are designed to force a dysbiosis rather than to mimic GF specifically.

Antibiotic depletion has the benefit of mimicking GF models in many ways. Generally speaking, a cocktail of poorly absorbable antibiotics are chosen that are either administered to mice through gastric lavage or supplementation of the drinking water[889]. Although completely sterilising animals is probably impossible, following treatment with the cocktail of antibiotics, depletion has been seen in the order of 20-400 fold reduction in bacterial DNA seen in feces[889, 890]. Phenotypically, these models are fairly effective at mimicking phenotypes seen in GF mice, including but not limited to the marked increase in caecum size[889-891].

Antibiotic depletion is significantly cheaper than GF to maintain. Although there is no specific definition of antibiotic depletion models, most studies describe the housing of these animals with standard techniques[520, 889-891], essentially requiring no extra equipment above and beyond what is required by a standard SPF animal facility. These models have been useful in microbiome research and have been instrumental in several important microbiome-host discoveries[520, 541, 892].

The critical issue with antibiotic depletion models is that they require large doses of antibiotics to be delivered consistently. These antibiotics, although they are usually chosen to be poorly absorbed in the GIT, may also have some direct effects mediated not by the depletion of the microbiome. For this reason, antibiotic depletion has not always mirrored

the effects seen in GF mice, and indeed host transcriptomics studies (although rarely performed for this purpose) of the gut in GF and antibiotic depleted mice demonstrated distinct expression patterns which may be related to either the remaining antibiotic-resistant microbes or ‘toxic effects' of the antibiotics themselves[893]. These models also leave the animals vulnerable to opportunistic pathogens, especially in the nonsterile environment that these animals are housed in, and for this reason, antibiotic regimes often include broad-spectrum antifungals in an attempt to prevent fungal infection[890] rather than specifically to reduce the burden of native fungal flora.

Antibiotic-induced dysbiosis models have also been well documented. The benefit of these models is that, unlike the depletion and GF models, these are the more analogous to human dysbiosis which is also likely to be caused by environmental perturbations. By virtue of this model's aims (specifically not to noticeably deplete the microbiome), there is significantly more latitude to select antibiotics for these models. Moreover, whilst the broader range of choice of agents is a benefit of this model, it also comes with the caveat that the specific antimicrobial chosen will have a specific characteristic effect on the microbiome and therefore findings must be examined in the context of the antimicrobial agent chosen. For example, butyrate-producing bacteria such as *Bifidobacterium* and *Lactobacillus* are susceptible to chloramphenicol, however, are usually resistant to metronidazole[894], meaning that their activity is likely to be impaired in a dysbiosis caused by chloramphenicol but not a dysbiosis caused by metronidazole. Essentially this means that unlike GF models, where the assumption that ‘all' effects of the normal microbiome are absent in GF mice, these dysbiosis models will have a spectrum of microbiome related effects dependent on the specific functional deficits involved. The implication, therefore, is that different dysbiosis models will demonstrate different potentially conflicting results. Antibiotic dysbiosis models, like depletion models[893], will also have antibiotic specific effects, independent of the microbiome, that must be considered in interpreting results. For these reasons, results must be interpreted with great care.

Infection Models

Infection models are much more varied and often much more specific. These rely on infecting an animal with a pathogenic organism which may or may not have broader effects on the composition of the microbiome. These models have been used to assess both specific

effects of infections and also more generally infection associated dysbiosis. More commonly used in microbiome studies of lower animals[895], the majority of the rodent literature referenced in this thesis have used other mentioned methods for assessing the role of the microbiome in physiology. Due to the individual specificity of each of these models, it is beyond the scope of this thesis to discuss them in detail. The biggest difficulty associated with these models from the perspective of appreciating the role of the microbiome is that phenotypes may be specific to the infection rather than dysbiosis and general.

Microbiome Transfer Models

Microbiome transfer models are also often highly specific, with specific microbiomes tested to determine the validity of a specific hypothesis. These models may seek to 'humanise' a GF or microbiome depleted mouse, to specifically monocolonise a GF with an individual microbe to determine its potential effects, or to assess the specific effects of the microbiome (usually collected from a disease model) in animals naïve to the treatment that induced the microbiome change noted. Humanised mice, referring to the establishment of human microbiome in mice, rely on GF or antibiotic depleted mice to provide the 'blank canvas' for which the microbiomes phenotype inducing efficacy can be assessed. There are two core assumptions inherent in these models. Firstly, it is assumed that the microbiome can interact with a phenotype of interest, and secondly that these effects can be recapitulated even after the microbiome has gone through the sampling, storage, and transfer processes. Interspecies microbiome transfer poses several issues as it is clear that many microbes viable in one species may not be viable in another species. Along these lines, the community composition also changes after transfer which is to be expected in animals with different biology, however it may be difficult to determine the role these changes have on the phenotype of the recipient.

The most useful effects of the transfer models have presented themselves in the multiple sclerosis literature. The microbiome in MS patients has been demonstrated to be relatively similar to healthy controls however it was through microbiome transfer models from people with MS that it has been shown that the microbes of these people impart a stronger effect in experimentally induced autoimmune encephalitis.

A within-species transfer also offers an understanding of the specific microbiome effects in the absence of the manipulation that caused the shift in the first place. The role of

the microbiome in nutrient intake efficiency was shown in elegant research by Turnbaugh *et al*[896]. In their research, they showed that the microbiome of genetically obese mice could induce excess weight gain in wild-type mice after transfer[896]. This finding exemplifies the role of intraspecies microbiome transfer models.

Monocolonisation models require GF animals as the basis for experimentation. Monocolonisation allows researchers to determine to the effect of a specific microbe in its interactions with the host. It allows for a functional assessment of individual microbes and may lead to probiotic drug discovery or to a greater understanding of the pathobionts frequently resident within the microbiome. Known pathobionts can also be used to determine the deleterious effects of dysbiosis as was shown by Sudo *et al*. in their initial paper assessing the microbiomes effect on the HPA axis. They found that GF mice had particularly abnormal HPA axis responses that could be partially resolved with monocolonisation of probiotic bacteria, however when monocolonised with enteropathic *E. coli*, the phenotype was worsened compared to GF[513] demonstrates that pathobionts can have further deleterious effects beyond their selfish actions within the microbiome.

1.6 Animal Models of Glaucoma

Animal models have significantly improved human understanding of illness. Human disease is generally challenging to study *in vivo* without significantly invasive assessment. Comparatively, animals can provide any tissue required at any timepoint allowing for in-depth analysis of cellular mechanisms, and therefore the potentially pharmacotherapeutic targets, of disease. Similarly, behaviour and disease phenotypes can be assessed macroscopically in animals, and the *in vivo* nature of disease development allows for the understanding of systems-based interactions that may occur in an illness. Animal models take many forms and attempt to mimic the biology of the human disease with as similar physiology as possible. Nevertheless, there are important considerations for each animal model, and these must be accounted for when interpreting any results from their use. Animal models may exist naturally or may be induced through manipulation of the animal's environment, through medication, or surgical intervention.

As the case may be, the majority of mammalian research occurs in rodents due, in part, to the logistical advantage of a small rapidly reproducing animal, and, in part, due to the relatively well-understood biology, and options for gene manipulation, to mimic genetic mutations in human physiology. Even so, glaucoma models have been evaluated in monkeys, dogs, cats, pigs, avians, and rabbits in addition to rodents[897].

Glaucoma is a pathology of RGC cell death, primarily caused by apoptosis. Naturally occurring glaucoma has been described in non-human primates[898], certain breeds of dogs[288, 899, 900], horses[901], and uncommonly in cats[902]. In rodents the inbred DBA/2J mouse line[903] and a model of congenital glaucoma in rats[904, 905] are the only spontaneous glaucoma models that exist.

1.6.1 Rodent Optic Nerve Injury Models

Glaucoma is a degenerative pathology of the optic nerve and therefore injuring the optic nerve has been used as a useful model of glaucomatous cell death. Optic nerve injuries can be performed *in vivo*, either surgically or through the administration of excitotoxic compounds[906].

Surgical optic nerve injury may take the form of ONC or optic nerve transection. Both these models involve the sedation and anaesthetising of a rodent, dissection of the conjunctiva and soft tissues behind the eye to visualise and then either crush or transect the

optic nerve. Care is taken not to disturb the intraocular vascular supply in these mice, which may be more difficult in optic nerve transection. These models are both very effective at causing RGC death with optic nerve transection causing a more profound effect on the RGC population[906]. Although these techniques can be technically challenging, the results of RGC death profile in these mice is highly reproducible, and therefore these represent a good model for assessing the potential for different agents/exposures to have even small effects on RGC survival[906]. Obviously, as these models are IOP independent, these can be used to directly assess how neuroprotective mechanisms may benefit the RGC loss seen in humans. ONC models vary in the amount of RGC death that occurs as the injury can be modified by both the magnitude of the force applied and length of time that the optic nerve is crushed[907]. Transection is much less modifiable and results in almost complete death of RGCs[906].

Standardisation of ONC procedures is an area of research that has been important for increasing the sensitivity of these models for use in research. To minimise the variability in the crush injury, there have been several techniques that have been suggested by various groups. The main variable being the method employed to deliver the optic nerve injury, methods have included using self-closing cross action forceps[907, 908], or aneurysm clips[908, 909] both of which offer a significant improvement on repeatability of the procedure from animal to animal when compared with standard forceps. Of these, cross action forceps induce a greater force on the optic nerve than aneurysm clips[908], and therefore these result in greater cell death in RGCs.

The benefits of these optic nerve injury models are clear. ONC/transection models are quick to perform, and the pathology is quick to develop[907]. These qualities allow these models to be used more rapidly for higher throughput experimental work. Since the injury to the optic nerve occurs primarily at the optic nerve head, and this appears to be the location of primary insult in human glaucoma, these models may mimic human pathology well.

The main drawback to these optic nerve injury models is that they initiate cell death in one swift insult, and although a progressive cell death occurs after the crush/transection, it is clear that there is a single initiating event. The single insult with a fairly coordinated and concurrent initiation of cell death in a large proportion of the RGCs is unlike human pathology where the dying cells undergo apoptosis at different time points over many years, with the vast majority of surrounding cells, at any particular timepoint, remaining healthy despite ongoing pathology[910].

The other two less commonly used optic nerve injury models are the intraocular injection of an excitotoxic compound[173] and ischaemia/reperfusion injury[906]. These models are much more specific to the glaucomatous processes that they model and are less commonly used as a general glaucoma model. Finally, tissue culture models are essentially an *in vitro* optic nerve transection model[345], but these remove the systemic interactions that *in vivo* models allow. As this thesis primarily concerns itself with the investigation of systemic interactions and their impacts on chronic glaucoma, these models are not discussed here.

1.6.2 Rodent Ocular Hypertension Models

The most commonly used models of ocular hypertension are either destructive or occlusive to the aqueous outflow.

One of the most commonly used methods is laser photocoagulation of the limbal and episcleral veins[897, 906]. These models all have the fairly consistent finding of rapidly increasing the IOP of treated eyes with a gradual return toward baseline over a variable timeframe[906]. Intraocular injection of microbeads, into the anterior chamber, has also been used to occlude the angle leading to chronic IOP elevation[906, 911]. These models seem to have a more consistent and more chronic elevated IOP[911] however their primary limitation is that they involve the injection of a foreign body into the eye which may be associated with inflammation of the eye[912].

Induced IOP models have a role in glaucoma research; however, there are broadly several limitations that must be considered in their use. Firstly, compared to optic nerve injury models, induced hypertension models are much less consistent in their effects on the retina as compared to ONC models. As these models are imperfect in the generation of a particular IOP, the error in the IOP generated is multiplied by the stochastic variation in the retinal resilience to IOP elevation across a batch of animals. Therefore the range in RGC losses seen after ocular hypertension models are typically larger than those seen after optic nerve injury[913, 914]. Secondly, inflammatory side effects of their treatments may impact on RGC loss outside of what may be expected to be seen as part of glaucomatous pathology. These considerations must be kept in mind when planning experiments using these models.

1.6.3 Genetic Models of Glaucoma in Rodents

The DBA/2J mouse line is perhaps one of the best utilised 'spontaneous' glaucoma models in the literature. DBA/2J mice develop progressive increased IOP from about 9 months of age, due to pigment dispersion in the anterior chamber leading to secondary angle closure, with subsequent loss of RGCs[915, 916]. The genetic cause of the glaucomatous phenotype in these mice appears to be mutations in *Tyrp1* and *Gpnmb*[917]. Some of the limitations associated with this model include the incomplete penetrance of glaucoma in this mouse, the somewhat unpredictable onset of disease, and relatively late onset of disease[914, 916]. A separate DBA/2 strain, the DBA/2NNia strain, has also demonstrated elevations in IOP with RGC loss, however, these mice experience loss of multiple other neural populations in the retina[918] and therefore these are less ideal for use in glaucoma research.

Other transgenic models have also been developed based on specific genetic associations seen in human glaucoma. A mutant myocilin mouse strain with a Tyr423His point mutation corresponding to the human mutation has been developed[919]. Mice have also developed with deficiencies or mutations in *Vav2/Vav3*[920], the collagen type I alpha1 gene[921, 922] (associated with primary congenital glaucoma[923]), *TBK1*[924] and *OPTN*[925].

1.7 Aims and Hypotheses

1.7.1 Research Question 1

Whereas the genome is a predictable and relatively concrete biological entity, the microbiome's plasticity and high interindividual variation make it a very complex system to describe. Simple quantitative assessments of its contents are already challenging to perform and remain imperfect due to their limited scope and kingdom. It is exceptionally clear that determining a qualitative assessment of the 'health' of the microbiome, i.e. to diagnose dysbiosis, is far from a precise science.

The concept of a pathomarker has been developed to help facilitate microbiome research even in the absence of very large longitudinal cohorts. A suitable pathomarker should be relatively easy to define and specifically associated with dysbiosis without significant and complex effect on the broader physiology that may impact on associations. IBS is a common illness that is readily identified in the community, with a consistent association to dysbiosis in the literature. IBS almost universally demonstrates a different taxonomic makeup of the microbiome in virtually every GIT microbiome that is assessed. IBS is often preceded by infection or antibiotic use, significant microbiome insults. There is growing evidence that microbiome manipulation may play an important role in future IBS therapies. For these reasons, it has been shown that IBS is suitable pathomarker of dysbiosis and its prevalence has been described in large longitudinal studies indicating that hypotheses, involving dysbiosis as a causative or contributing factor, can be analysed 'prospectively'.

IBS is especially useful in assessing a link between dysbiosis and glaucoma as the two illnesses are so separate in their physiology. The risk factors for IBS and for Glaucoma have minimal crossover. There is no known biologically plausible mechanism to link IBS to glaucoma through the effects of IBS on mental health. Similarly, although neuroinflammation is a factor, investigated frequently, in glaucoma, it is quite possible that microbiome effects on the immune system may play a role in any association found.

This leads to the first research question: Are adults with IBS more likely to develop glaucoma? People with IBS had 1.46 fold increased hazard for developing PD[926] and 1.76 fold increased hazard for developing AD[570]. Given these relationships, an effect size of 1.5 was hypothesized for the relationship between IBS and glaucoma.

Research Aim 1

To quantify the prevalence of IBS in an Australian cohort of glaucoma sufferers as compared to the general Australian adult population

Hypothesis:

Australians with advanced glaucoma will be 1.5 times as likely to also have IBS as an age and gender matched cohort of regular Australians.

Research Aim 2

To identify and quantify an increased rate of glaucoma in adults with IBS in two large population based European cohorts.

Hypothesis:

In two very large population based European cohort studies, adults with IBS will be associated with 1.5 times increased odds of developing glaucoma over the course of the follow up of both studies.

1.7.2 Research Question 2

Dental illnesses such as caries and periodontitis are also associated with abnormal microbiome. Although the causal chain for both dental illnesses is less clear, these there are bidirectional effects between the immune system and the oral microbiome that contribute to the development of these illnesses. Indeed, there is some research to suggest that antiseptic mouthwash, and antibiotics may play a role in these illnesses suggesting that there may a reversible component to these illnesses specifically caused by microbiome disturbances. These may both manifest in a loss of teeth, and therefore, although less clear than IBS, oral health issues present another pathomarker for dysbiosis. Prior to beginning this research there had also been some limited research by other to suggest that the oral microbiome was associated with glaucoma in an African American population.

This leads to the second research question addressed in this thesis: Are dental illnesses associated with increased risk of glaucoma?

Research Aim 3

To identify and quantify the size of an association between dental illness (periodontitis and incidental tooth loss) and the incidence of glaucoma in a large cohort of male US American healthcare workers.

Hypothesis:

In a large cohort of male American health professionals, people with periodontitis, or incidental tooth loss, will have 1.5 times increased odds for developing glaucoma.

1.7.2 Research Question 3

BDNF is a neuroprotective compound with effects on glaucomatous optic nerve damage. The microbiome has been implicated in BDNF modulation in a number of CNS areas including the hippocampus, the prefrontal cortex, the cingulate cortex, the brainstem, the hypothalamus, and the amygdala. Although there is yet no data assessing the retina and its regulation of BDNF in response to the microbiome, it is possible that the underlying mechanism that appears to cause this almost pan-CNS effect also plays a role in BDNF modulation in the retina.

The mechanism linking the microbiome to BDNF modulation has not been definitively proven. Initially, there was a suggestion that the vagus nerve was responsible for the majority of gut-brain interactions however it's not clear from the literature that this is the case. The literature includes a report demonstrating that vagotomy did not appear to affect the interaction between the microbiome and BDNF expression, suggesting that these effects were caused by some other pathway. If the effect is mediated by circulating compounds, the BBB, which is itself under some manipulation by the microbiome, may play a role in access to the CNS although this may also be affected by the microbiome. Until a circulating chemical or synaptic neural mechanism can be identified for the effect seen, the potential for the microbiome to impact on any particular group of CNS neurons cannot be ruled out, without experimentation.

Although there are several microbiome manipulation models, GF mice (when compared to SPF and conventionalised) are the best model for assessing the beneficial aspects of the normal microbiome. These mice specifically help to reveal the homeostatic mechanisms that the microbiome is involved in, indicated by any deficits/phenotypic differences when compared to mice with normal microbiomes.

The ONC is an acceptable model to assess the microbiome mediated neuroprotective effect at the optic nerve. Given the precision with which the model can be performed and the speed at which results can be garnered, this model offers significant benefits to this experimental suite than hypertensive glaucoma models. Similarly, as ONC is a gentler model

than optic nerve transection, it is likely this model allows for more room to differentiate cell survival between experimental groups.

This leads to the third research question addressed in this thesis: Does the microbiome protect RGCs in an ONC model of glaucoma? if so, is this effect mediated by BDNF?

Generally speaking, BDNF levels, depending on the brain region of interest have been found to be approximately 30% lower in GF mice than in SPF mice[513, 516], and so this was hypothesised as the expected difference in the retina. Similarly, in a glaucoma model treated with low dose BDNF supplementation, cell survival was about 20% less in untreated eyes[348], which was therefore hypothesized as the effect that would be seen in GF mice.

Research Aim 4

To quantify the rate of RGC death after a model of ONC in GF, SPF, and CON mice.

Hypothesis

By day's 7 and 35, after ONC, GF mice will have 20% less RGC cell survival relative to SPF mice at the same timepoint, and that CON mice will have similar cell survival to SPF mice.

Research Aim 5

To quantify the expression of BDNF in the retinae of GF, SPF and CON mice, at baseline and after ONC.

Hypothesis

GF retinae will have 30% less BDNF than the retinae of SPF and CON mice, and that this difference will increase further by day 3 after ONC.

Chapter 2 – Host-Microbe Interactions in the Central Nervous System

2.1 Chapter Overview

In the previous chapter a detailed assessment of the literature linking the microbiome to the CNS was displayed. The following chapter is a narrative review that suggests the Aryl Hydrocarbon Receptor (AHR) is an important link between the microbiome and its effects on the CNS. The review presented here looks at the topic of host microbiome communication from both a neurodevelopmental and neurodegenerative perspective. The effects of the microbiome and the AHR are analysed in tandem with the view to drawing comparisons and developing the hypothesis that the AHR may play a role in mediating microbiome host interactions.

The central theoretical model of the presented work is the cohesion of the holobiont and therefore the authors of this paper recognise that the AHR may be only one of many communication mechanisms functioning between the host and its microbiome. As will be discussed in the article, the AHR is vital to the function and maturation of glial cells, particularly microglia which are significant in the neuroinflammatory processes of neurodegeneration. Neuroinflammation in the pathogenesis of glaucoma has already been discussed in '1.1.3.1 The Role of Inflammation'.

In addition to the development of the idea of AHR as a communication pathway between the microbiome and the host, this article also explores how the microbiome is responsible for functionality in both neurodevelopment and neurodegeneration, and with respect to Autism Spectrum Disorder and Ischemic stroke.

This review was published in the Journal of Molecular Medicine. I was co-first author with Dr Hae-Ung Lee, listed second alphabetically. The paper was submitted 5 August 2016, revised 31st October 2016, and accepted on 3rd of November 2016. The citation for this paper is as follows.

Lee HU, McPherson ZE, Tan B, Korecka A, Pettersson S. Host-microbe interactions: The aryl hydrocarbon receptor and the central nervous system. J Mol Med 2017:95(1);29-39

Permission to reproduce this paper here, may be found at Appendix 1. The figures, tables, and references have been renumbered in line with the formatting of this thesis.

2.2 Abstract

The microbiome located within a given host and its organs forms a holobiont, an intimate functional entity with evolutionarily designed interactions to support nutritional intake and reproduction. Thus, all organs in a holobiont respond to changes within the microbiome. The development and function of the central nervous system and its homeostatic mechanisms is no exception and are also subject to regulation by the gut microbiome. In order for the holobiont to function effectively, the microbiome and host must communicate. The aryl hydrocarbon receptor is an evolutionarily conserved receptor recognizing environmental compounds, including a number of ligands produced directly and indirectly by the microbiome. This review focuses on the gut microbiome-brain axis in regards to the aryl hydrocarbon receptor signalling pathway and its impact on underlying mechanisms in neurodegeneration.

2.3 Introduction

The alimentary tract contains trillions of microbes with overlapping biological and biochemical needs due to co-evolutionary mechanisms, collectively termed the gut microbiome. Though researchers have shown that the gut microbiome impacts virtually all aspects of host function, the mechanisms and signalling pathways by which the gut microbiota communicates with its host are still unknown.

Bacteria and archaea, two of the predominant kingdoms within the microbiome, were the dominant forms of life on Earth for approximately 3 billion years prior to the evolution of the animal kingdom [927, 928]. Current understanding increasingly considers the host and its microbiome as a working functional unit known as the holobiont. Environmental changes affect both the host and its microbiome. The last decade of genome-wide association studies has ignored the microbiome and, consequently, missed the response elicited within it. In the last 20 years, germ-free (GF) mice, mice that are raised without exposure to any microbes, have been used to address the holobiont concept using a systems biology approach [929]. A prerequisite for a holobiont to function is the ability of the host and microbiome to communicate, to maintain homeostasis and act correspondingly when exposed to assaults. We postulate that many of the ligands and receptors identified and used for microbiome-host interactions are evolutionary. This review focuses on the well-described xenobiotic aryl hydrocarbon receptor (AHR) as one possible evolutionarily conserved signaling pathway that contributes to microbiome-host homeostasis within the holobiont.

2.4 The Aryl Hydrocarbon Receptor

The AHR is a cytoplasmic ligand-induced receptor originally discovered as a xenobiotic sensor mediating the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), also known as dioxin [930-933]. The metabolism of xenobiotic compounds is initiated by activation of the AHR, which then translocates to the nucleus, where it acts as a transcription factor for specific target genes, such as cytochrome P450 1A1 and cytochrome P450 1B1 [930, 931, 934-938]. However, invertebrates do not have a toxic response to dioxin, and none of the currently known invertebrate AHR orthologues, including spineless in Drosophila, have dioxin binding capacity, which suggests that the ancestral role of the AHR is not specifically toxin response [939, 940]. Furthermore, physiological roles of the AHR in responses to endogenous ligands have been reported in cell cycle regulation, cell differentiation, and immune responses [937, 941-944]. A number of endogenous AHR ligands have been suggested through *in silico* research and biological testing, including tryptophan metabolites[931, 937, 945]. Recently, our group discovered that AHR expression is attenuated in GF mice[946]. This finding suggests that the AHR acts as a mediator in communication between the host and gut microbiota.

2.5 Function of the Aryl Hydrocarbon Receptor in Host-Environment Interactions

Dioxin-activated AHR attenuates lipid metabolism via negative regulation of peroxisome proliferator-activated receptor (PPAR)[947]. Dysregulation of lipid metabolism leading to hepatic steatosis and insulin resistance suggests that the AHR plays an important role in integrating exogenous and endogenous influences in lipid and energy metabolism[948, 949]. Findings from AHR-deficient mice show that, like GF mice[950, 951], they are protected from high fat diet-induced obesity, hepatic steatosis, and insulin resistance[952].

Recently, fibroblast growth factor 21 (FGF21) was reported to be a novel target gene of the AHR. FGF21 increases lipid oxidation and ketogenesis but decreases gluconeogenesis at the gene expression level[953, 954]. As an insulin sensitizer, FGF21 boosts the metabolic benefits such as improved blood glucose levels due to increased glucose uptake in adipocytes, reduced body weight due to increased energy expenditure, and improved blood lipid profiles due to hepatic sequestration of lipid droplets[955-957]. TCDD-induced AHR activation has been shown to increase FGF21 mRNA in both a dose- and time-dependent manner in mouse liver [948, 949]. In addition, drug-induced over-expression of human AHR in mice induces the activation of FGF21 which may then result in decreased insulin resistance[958]. The opposite effects were observed with the down-regulation of FGF21 – insulin insensitivity, deranged lipid profile, and liver inflammation – and can be associated with the attenuation of hepatic lipid accumulation and increased transfer of fats out of the liver in hepatocyte-targeted AHR knockout (KO)[948].

Recent work from our lab linked the mechanism of microbiota and host communication through an AHR-dependent mechanism. We demonstrated that the AHR is differentially expressed in GF mice. Similarly, our AHR-KO study showed that AHR regulates a set of metabolic genes in the liver, including CD36 (involved in fatty acid uptake) and Hmgcs2 (an enzyme involved in ketone body regulation)[946]. Similar to fast-induced adipose factor-KO mice[951], AHR-KO mice gain weight as expected but do not develop insulin resistance[946], suggesting that AHR could be the upstream link between microbiota-mediated signals and the host[946].

*S*everal reports have associated AHR function with the regulation of the immune system. TCDD treatment has shown that AHR has the capacity to mediate the differentiation and/or function of T cells, macrophages, and dendritic cells[936, 938, 941, 959-963]. The activation of AHR by TCDD[964-966] and the ablation of AHR in KO animals[967] has implicated this receptor in viral immunity. We also recently reported that ablating the AHR in $CD11c^{+}$ cells perturbs the

development of the intestinal epithelium and intestinal immunity[968]. Depending on the presence of specific ligands, AHR activation has also been shown to suppress or exacerbate responses in experimental autoimmune disease models. For example, TCDD and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) can suppress experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS)[961], whereas the activation of AHR by ligands such as 6-formylindolo[3,2-b]carbazole (FICZ) exacerbates the development of EAE[942, 961, 963, 969]. In addition, the affinity of AHR for ligands (TCDD, high affinity; FICZ, low affinity) influenced the amount of IL-17 and IL-22 protein secreted by Th17 cells[970]. These findings indicate that various ligands for AHR may have different effects on host development.

2.6 Natural Ligands for the Aryl Hydrocarbon Receptor

Though most research on AHR has focused on man-made high affinity binding ligands and chemical pollutants, recent research has implicated important roles for an array of low affinity natural ligands produced, metabolized, or influenced by the gut microbiota. Natural ligands for AHR can be divided into three groups: host mediated, microbiota mediated, and dietary (Figure 2.1).

The essential amino acid tryptophan is the major source for both host-mediated and microbiome-mediated AHR ligands. Kynurenine (KYN) is converted from tryptophan by tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) and is an important AHR ligand[970]. Kynurenic acid (KYNA) is converted from KYN by kynurenine aminotransferase and also an important ligand[971]. Our research has shown that the microbiota regulates the expression of IDO in the liver, and although IDO may play a more important role in KYN metabolism in extra-hepatic tissue[972], these results indicate a need to analyze the role of the microbiota in KYN metabolism[946].

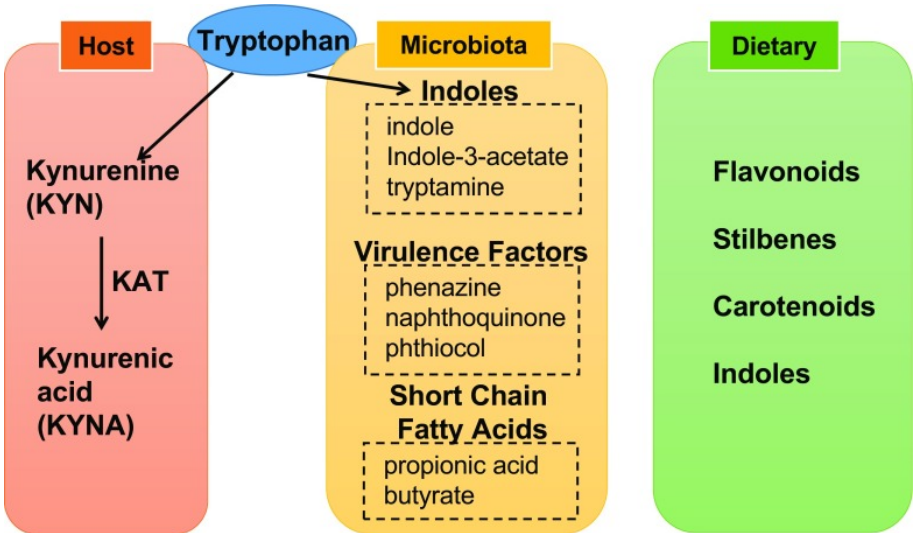


Figure 2.1: Natural ligands for aryl hydrocarbon receptor.
Kynurenine (KYN) is converted from tryptophan in host metabolism. Kynurenic acid (KYNA) is also an AHR ligand, converted from KYN by kynurenine aminotransferase (KAT). There are three groups for microbiota-mediated AHR ligands: (1) tryptophan metabolites derived by microbiota, (2) bacterial virulence factors, and (3) short chain fatty acids. Short chain fatty acids are not direct ligands for AHR, but those facilitate AHR effects. Flavonoids, stilbenes, carotenoids, and indoles from plants are dietary ligands for AHR

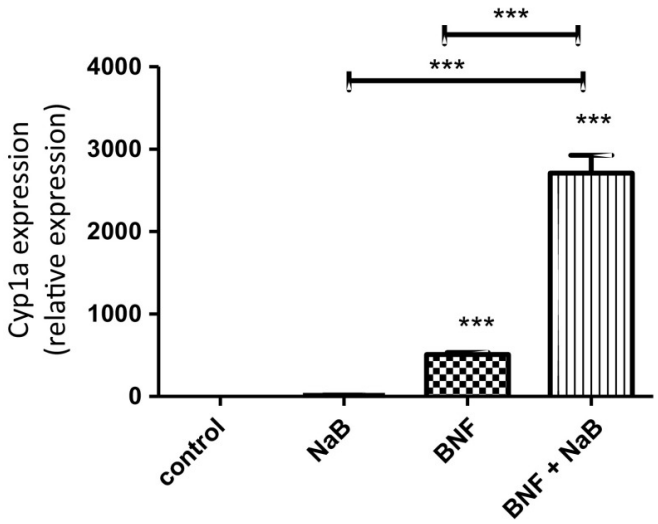


Figure 2.2: Sodium butyrate (NaB) increases the activity of the aryl hydrocarbon receptor. HepG2 cells were cultured for 24 h with normal media or media containing NaB, beta-naphthoflavone (BNF), a natural agonist for AHR or NaB and BNF. While NaB not having a direct ligand effect on AHR demonstrated a significant synergistic effect to increase the activation of cyp1a by BNF

Gut microbiota also convert tryptophan to indole, indole-3-acetate, and tryptamine, which have been identified in mouse and human intestine and work as AHR agonists and antagonists[973, 974]. Microbial pigment virulence factors, namely the phenazines from microbes such as *Pseudomonas aeruginosa* and the naphthoquinone phthiocol from *Mycobacterium tuberculosis*, act as microbiota-mediated AHR ligands. Upon ligand binding, AHR activation leads to virulence factor degradation and regulates cytokine and chemokine production[975]. Short chain fatty acids, such as propionic acid and butyrate, from the microbiome are not direct ligands for AHR, but our recent data suggest that they stabilize AHR, increasing its activity in the presence of true ligands (Figure 2.2).

The majority of dietary AHR ligands are produced by plants. Plant-derived compounds that act as ligands for AHR include flavonoids, stilbenes, carotenoids, and some indoles. Indole-3-carbiol (I3C) is an indole compound found in cruciferous vegetables that is converted to higher affinity AHR ligands, such as indolo-[3,2-b]-carbazole and 3,3'-diindolymethane in the acidic environment of the stomach[976].

2.7 Microbiome and Neurodevelopment

An important aspect of the functionality of the holobiont is the ability of each component to shape the behaviors of the others. Simply put, it behooves the microbiota to encourage certain 'healthy' behaviors in the host. Although the effects of the microbiota are important in maintaining metabolism and the immune system, it is logical to conclude that the microbiota, acting in the best interests of the whole holobiont, may play a pivotal role in supporting the development of the central nervous system (CNS).

2.7.1 The Microbiota and Neurodevelopment

Sudo *et al*. first demonstrated a possible link between the hypothalamic-pituitary-adrenal (HPA) axis and the gut microbiome[513]. Elevated adrenocorticotropic hormone and corticosterone levels were observed in GF mice compared to specific pathogen-free (SPF) mice in early life. They also demonstrated that brain-derived neurotrophic factor (BDNF) is significantly reduced in the hippocampus and cortex of GF mice[513]. Later studies confirmed regulation of steady state levels of BDNF by the microbiome[515, 516], which plays an important role in neuroplasticity, neuron differentiation, and the maintenance and protection of neurons under stress. Many of these groups have linked changes in brain biochemistry to altered behaviors in GF mice[513, 515, 516].

Recently there have been reports that the microbiome plays an important role in the growth and function of CNS cell populations. Hippocampal neurogenesis was shown to be increased in GF mice[977], which also correlated to increased volume and abnormal neuronal morphology in the hippocampi of GF mice[978]. Similarly there was increased amygdala volume in GF mice with concomitant neuronal morphology[978]. In contrast to this, hippocampal neurogenesis was shown to be decreased in mice treated with antibiotics[979]. Möhle *et al.* demonstrated that their model of antibiotic depletion lead to decreased hippocampal neurogenesis through modulation of the populations of specific immune cells[979]. Our group have also reported that the microbiome plays a key role in the maintenance of other synaptic proteins, including synaptophysin and PSD-95, both of which are reduced in the striatum of SPF mice, suggesting abnormally hyperactive synaptogenesis in the striatum of GF mice[515]. The microbiome has also been implicated in the functionality of glial cells. Our group have demonstrated that the microbiota is instrumental in the development of the blood-brain

barrier (BBB)[555]. Finally, the Hoban *et al.* demonstrated that in the absence of microbiota, there is increased myelination of neurons in the pre-frontal cortex[980].

Though these observations are of interest, knowledge on the molecular mechanisms linking the microbiome to neurodevelopment remains limited.

2.7.2 The Aryl Hydrocarbon Receptor in Neurodevelopment

Reports on the AHR in neurodevelopment are very limited. However, the AHR appears to be vital in the maintenance of some key pathways in neurodevelopment in worms. In *Caenorhabditis. elegans*, Huang *et al*. demonstrated a role for AHR in neural cell fate determination, particularly for GABAergic neurons[981]. Ahr-1 is the AHR orthologue in *C. elegans*. In worms with *ahr-1* mutations, two specific neurons out of the 302 total neurons have been reported to appear and act like a second pair of neurons that could be reprogrammed into the first pair of neurons by ectopic administration of ahr-1[981]. In addition, Qin *et al*. found that *ahr-1* is responsible for the development, orientation, and axonal migration of ahr-1-expressing neurons in *C. elegans*[982]. Taken together, these results demonstrate that AHR contributes to the cell fate determination of specific neuronal populations in worms, possibly through natural ligands and irrespective of dioxin exposure.

Dioxin toxicity studies have demonstrated that the AHR is likely to play a role in CNS development. In zebrafish, TCDD exposure was reported to reduce the total number of neurons by 30%[983]. In mice, dioxin toxicity studies have demonstrated a similar role for AHR in the embryonic differentiation of GABAergic neurons in the telencephalon[984] and the neurogenesis of cerebellar granule cells[985]. Importantly, due to the extraordinarily high binding affinity of dioxin for the AHR, emphatic conclusions regarding the physiological role of the AHR in normal development cannot be drawn from dioxin studies alone.

The AHR was also shown to play a crucial role in CNS development in studies more consistent with typical biology. The expression of a constitutively active AHR in mice retarded the development of interneurons in the olfactory bulb[986]. Furthermore, in mouse primary cortical neurons, AHR activation by FICZ was also shown to increase the expression of synaptophysin and SAP102, but not PSD95[987]. In functional experiments, the AHR was shown to alter hippocampal neurogenesis and contextual fear memory in mice[988], as well as aggression behavior in *C. elegans*[989]. Latchney *et al.* demonstrated that adult AHR-KO mice and TCDD-exposed mice hippocampal-dependent memory impairment. AHR-deficient mice

and TCDD-exposed mice also exhibited reduced cell proliferation, survival, and differentiation in the adult dentate gyrus[988]. The often conflicting data demonstrating both the KO and activation of AHR lead to similar outcomes, suggesting that the AHR plays a vital role in CNS homeostasis.

2.7.3 Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a neurodevelopmental illness for which evidence supports a possible link between the maternal/early postnatal microbiome and dysfunctional neurodevelopmental programming. From a human health perspective, the association between the microbiome and neurodevelopment was highlighted by evidence that people suffering from ASD also frequently present with problems related to a dysfunctional bowel with aberrant intestinal barrier function[990]. Although the association remains controversial, a role for dysfunctional gut microbiome-brain axis has gained further support from the recent demonstration of different microbiome composition in children with ASD compared to age-matched controls[991].

A recent study demonstrated that, in an animal model of ASD, correction of the microbiota with probiotic administration of *Bacteroides fragilis* corrected biochemical and behavioral abnormalities associated with ASD[992]. In this ASD mouse model, the key effector in the microbiota-gut-brain axis was the metabolome; a number of specific metabolites altered in the ASD mouse model were normalized by the treatment. Indolepyruvate, a microbially controlled molecule that is metabolized into an AHR agonist, was significantly regulated in the ASD model and by *B. fragilis* treatment[992]. This metabolite is an interesting corollary to indolyl-3-acryloylglycine, which has been shown to be elevated in the urine of humans with ASD[993].

Epidemiological studies of Vietnamese children exposed to TCDD in the prenatal and perinatal period have demonstrated increased neurodevelopmental defects and autistic traits in children with greater exposure to TCDD[994]. Prenatal and postnatal exposure to KYN in rats causes cognitive defects in adulthood[995]. Although Pocivavsek *et al*. did not identify a specific mechanism underlying the association between early life KYN exposure and cognitive deficits, they did note that the treatment led to 3.4- and 2.1-fold increases in KYNA levels in the brain at postnatal days 2 and 21, respectively[995]. Although Pocivavsek *et al.* noted the effects of KYNA as an antagonist of the α7 nicotinic acetylcholine receptor and the N-methyl-d-aspartate

receptor[995], KYNA is also an AHR ligand with a stronger binding affinity for the AHR than KYN[971], potentially implicating AHR activity in the cognitive abnormalities observed in this model.

An animal model of ASD appearing to be caused, in part, by microbial metabolites that act on the AHR and epidemiological studies linking environmental exposure to AHR ligands to neurodevelopmental issues and strong associations between ASD and gastrointestinal pathology, suggest that ASD is a systems biology problem within the holobiont. Therefore, the AHR signaling pathway and its microbially derived natural ligands are of great interest for further exploration of ASD and other neurodevelopmental disorders.

2.8 Neurodegeneration

Neurodegeneration is regarded as a pathological process whereby neuron loss is increased, frequently in association with aging. The mechanisms underpinning neurodegeneration and neuron loss are poorly understood, but are assumed to be the result of a metabolic dysfunction, increased autophagy, and aberrant host immune system activity.

Irritable bowel syndrome (IBS), an illness associated with disruption of the microbiota, has been shown to be a risk factor for Parkinson's disease[926] and both non-Alzheimer's disease dementia and Alzheimer's disease[570]. Our group also recently showed that IBS may precede glaucoma, a progressive neurodegeneration of the optic nerve, in two primarily Caucasian populations[996]. These results provide evidence that pathological mechanisms underlying IBS, including disruption of the microbiota, may have clinically relevant effects in neurodegenerative illnesses and alter homeostatic mechanisms in the CNS. Moreover, tryptophan metabolism by the microbiota has been suggested to play a role in IBS pathology through AHR-mediated pathways[997].

Parkinson's disease, which has long been known to be associated with gastrointestinal dysfunction, has been theorized to be initiated within the gut and follow a prion-like spread of pathology through the vagus nerve into the brain[998]. The effects of microbiome-driven inflammation on Parkinson's pathology were assessed by orally administering bacterial lipopolysaccharide, which caused a rapid increase in alpha synuclein expression in the myenteric neurons of the mouse gut[999]. In humans, Parkinson's disease is associated with alterations in the microbiota, particularly with regards to Prevotella and Enterobacteria[1000].

Finally, an interesting pre-print article has demonstrated that the microbiome may play a role in the formation of Beta-Amyloid plaques in the mouse brain. GF Alzheimer's transgenic mice demonstrated significantly lower levels of Beta-Amyloid in the brain than conventionally raised transgenic mice. Moreover, the faecal 16S RNA analysis showed that Alzheimer's transgenic mice had a significantly different microbiome to wild type mice and faecal transplants from transgenic mice but not wild type mice was able to significantly upregulate the Beta-Amyloid in the brains of Germ Free Alzheimer's transgenic mice[571].
The assessment of the microbiota in patients with neurodegenerative illnesses is ongoing.

2.8.1 The Blood-Brain Barrier

The AHR is widely expressed in the CNS[1001, 1002]. However, our understanding of the role of the AHR in neurons and supporting cells is still very limited. The BBB is vitally important in the maintenance of CNS homeostasis and its weakening has been suggested to contribute to neurodegenerative pathology. Breakdown of the BBB at the hippocampus has been correlated with cognitive impairment in humans[1003]. Previously, our group reported that the BBB exhibits increased permeability in adult GF mice[555]. Mono-colonization with *Clostridium tyrobutyricum* or *Bacteroides thetaiotaomicron* and treatment with sodium butyrate had rescuing effects on BBB permeability and tight junction protein expression[555]. One mechanism of the microbiome-mediated effects on BBB permeability appeared to be related to changes in the expression of tight junction proteins, such as occludin and claudin-5[555]. A recent report demonstrated that induction of dysbiosis with a mixture of antibiotics caused alterations in the mRNA expression of tight junction proteins in the brain[521], validating, at an mRNA level, the results produced by Braniste *et al.* in a separate model of microbiome disruption.

The presence of the AHR and expression of its target genes has been shown to be significantly elevated in the microvessels of the brain[1002, 1004]. Contradictory results have been reported. Via activation by TCDD, the AHR decreases the permeability of the BBB *in vivo*[1005, 1006], but increased BBB permeability was observed following exposure to 3-methylcholanthrene[1007]. Interestingly, though the increased BBB permeability reported by Braniste *et al*. has not been assessed in the context of the AHR, a recent study in keratinocytes demonstrated that ligand activation of the AHR elevates occludin and claudin 1 and 4[1008], indicating that a similar AHR-mediated effect could occur in the BBB. One of the most abundant gap junction proteins in the BBB is connexin 43. Connexin 43 expression and gap junction integrity has been shown to be down-regulated by AHR activation[1009, 1010]. The deletion of connexin 43 is known to weaken the BBB, allowing it to open under increased vascular hydrostatic pressure or shear stress[1011]. A recent report suggested that connexin 43 is integral to brain immune quiescence[1012] and, irrespective of BBB integrity, the deletion of connexin 43 was associated with increased immune cell recruitment across the BBB. Moreover, deletion of connexin 43 leads to activation of the endothelium and chemoattraction, thereby linking a key molecule in the maintenance of BBB integrity with the neuroinflammatory response[1012].

2.8.2 Neuroinflammation

The role of the AHR in the immune system is being increasingly appreciated[1013], and the role of neuroinflammation in psychiatric diseases is also being recognized[1014]. One of the hallmarks of neuroinflammation that potentially impacts the neuropsychiatric phenotype[1015] and neurodegenerative pathology[1016] is the chronic activation of microglia. GF mice have immature microglia with unusual activation properties[540]. Furthermore, microglia from GF mice have altered gene expression profiles similar to the SOD1 mouse model of amyotrophic lateral sclerosis[1017]. Although some of the GF microglial phenotypes could be rescued by short chain fatty acid supplementation[540], this does not preclude the possibility of microbiotic interactions through alternate pathways, including the AHR.

AHR mediates both pro-inflammatory and anti-inflammatory effects in microglia[1018]. Lee *et al*. found that AHR activation with FICZ and 3-methylcholanthrene attenuates microglial immune responses. They also demonstrated that silencing the AHR gene with siRNA reduces microglial activation, demonstrating a pro-inflammatory effect of the AHR[1018]. Other groups have found similar pro-inflammatory and anti-inflammatory effects of the AHR. Within the CNS of AHR-null mice, microglia accumulate in the retina in a model of age-related macular degeneration[1019].

Dietary and microbiotic metabolites, particularly tryptophan metabolites[938], may play an important anti-inflammatory role in the CNS. FICZ was recently shown to modulate astrocyte activity and CNS inflammation through the AHR[1020], thereby linking the microbiota directly to neuroinflammatory mechanisms through the AHR. Astrocytes are the most abundant glial cell population in the CNS, participating in metabolism, neuronal transmission, and inflammation[1021, 1022]. In a mouse model of CNS autoimmunity, CNS inflammation induced a type 1 interferon-mediated response in astrocytes, which induced AHR activation[1020]. This AHR response was shown to limit astrocyte inflammation and was increasingly efficacious when mice were supplied dietary tryptophan. To demonstrate that the effects were due to microbiota-mediated metabolism of tryptophan, ampicillin (a broad spectrum antibiotic) was given, which interfered with the effects of dietary tryptophan, and the treatment of mice directly with indoxyl-3-sulfate (a microbial metabolite of tryptophan) led to AHR-mediated anti-inflammatory effects[1020]. The dietary metabolite and AHR ligand indirubin-3'-oxime was also shown to inhibit the inflammatory activation of microglia in the rat brain[1023]. Whether the immune system regulatory systems exhibited by the AHR in the periphery are relevant to

neuro-inflammatory responses is not clear. Moreover, as different ligands have different effects on the transcriptional effects of the AHR, the effects of endogenous and exogenous AHR ligands require deep investigation to further elucidate the varying effects of the receptor in the regulation of neuroinflammation.

The pro-inflammatory and anti-inflammatory effects of the AHR are likely due to the complex interactions the receptor can have with other transcription factors. For example, the pro-inflammatory cytokine TNF-alpha is up-regulated when microglia are stimulated by lipopolysaccharide, but this effect is attenuated both when the AHR is activated by FICZ and when the AHR is silenced by siRNA due to the complex interactions between the receptor and NF-κB, which can be modulated by the application of AHR ligands[1018]. Similarly, Rothhammer *et al*. found that the AHR-mediated anti-inflammatory effects in astrocytes are due to the limitation of NF-κB activation[1020].

2.8.3 Ischemic Stroke

One interesting neurodegenerative process clearly regulated by the microbiota and microbiota-metabolized AHR ligands is ischemic neurodegeneration. In three separate mouse models of microbiota disruption, the microbiota was shown to impact the outcome of ischemic stroke[542, 892, 1024]. Depletion of the microbiota with a cocktail of antibiotics decreased survival in a middle cerebral artery occlusion (MCAO) model of murine stroke and severe colitis in mice after stroke. Interestingly, that study found no significant difference in infarct size 1 day after stroke[892]. In a separate model in which the microbiota of mice was altered, not depleted, with amoxicillin and clavulanic acid, infarct volume was significantly reduced compared to mice with a healthy microbiota[542]. This microbiota-stroke effect may be bi-directional, as Singh *et al*. demonstrated that particularly large infarcts can cause dysbiosis within the microbiota, possibly potentiating neuroinflammatory effects within the CNS[1024]. Benakis *et al.* demonstrated that intestinal IL-17^{+} γδ T cells, which were reduced in their model of dysbiosis, accumulate in the meninges after stroke and are responsible for a neuroinflammatory response that potentiates damage after ischemic insult[542]. Interestingly, the AHR alters the function of γδ T cells, and its stimulation with FICZ elevates IL-17 production in these cells[1025]. In human illness, elevated serum levels of KYNA during the acute phase of stroke has been correlated with worse neuropsychiatric outcomes in stroke patients[1026]. Similarly, IDO activity, as determined by the KYN to tryptophan ratio, is positively

associated with stroke severity[1027], and other elements of the KYN metabolic pathway have been correlated with infarct size in stroke[1028]. To evaluate the specific role of the AHR in stroke, Cuartero *et al*. used the MCAO stroke model to demonstrate that the receptor is up-regulated and activated after ischemic insult[1029]. Pharmacological inhibition of the AHR resulted in a smaller infarct size and greater functional outcomes, and stimulation of the AHR resulted in increased infarct volume[1029]. KYN levels in the brain were also elevated in this model of stroke and, through activation of the AHR, play a deleterious role in cerebral ischemia[1029]. How the microbiota interacts with tryptophan metabolism to affect the AHR in neural ischemia is still unknown.

2.9 Summary and Further Directions

As mounting evidence supports the holobiont model of the host and its microbiome, one of the most important questions facing researchers are the mechanisms by which the microbiota communicates with the host. The AHR is an evolutionarily conserved ligand induced receptor involved in host-environment interactions. Despite its well-known responsiveness to man-made compounds, such as TCDD, in invertebrates the AHR does not elicit a response to dioxin. Therefore, AHR must execute other evolutionarily important roles in development and homeostasis. Interestingly, we and others have found that AHR responds to microbiome-mediated ligands engaging host immune and metabolic responses. Moreover, many AHR ligands cross the BBB, implying a role of AHR in the CNS (Figure 2.3). While preparing this review, we have realized that there are much more to be learned about the AHR signaling pathway and its impact on CNS development and function. The pleiotrophic action of AHR and its wide expression pattern may also hold hope for the development of new microbiome derived compounds that support the metabolic homeostasis within the holobiont.

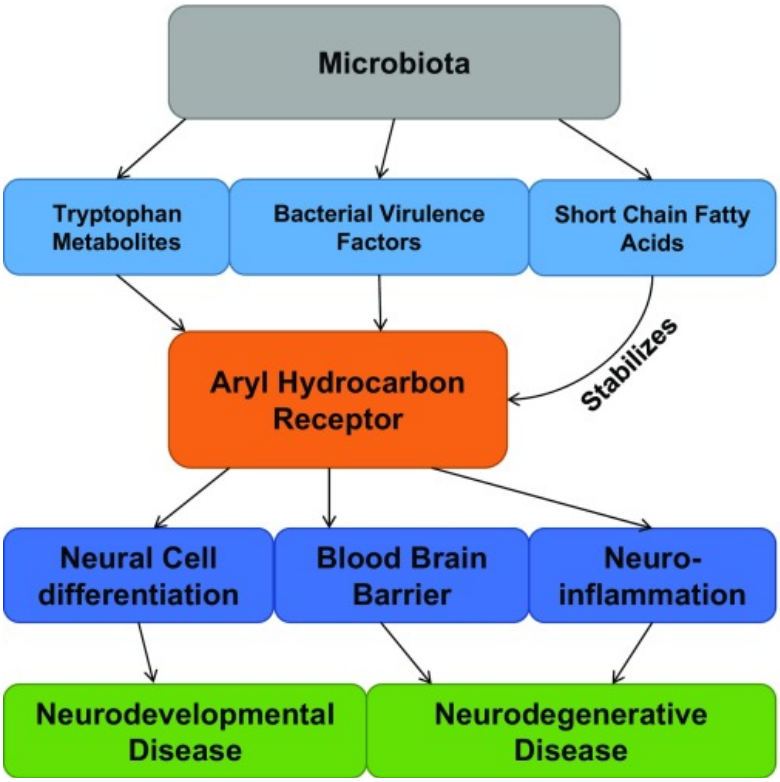


Figure 2.3: Proposed model. The activities of the microbiota through the Aryl Hydrocarbon Receptor (AHR) on the central nervous system

Section 2 – Epidemiological Research

Chapter 3 – Adults with Glaucoma are More Likely to also Have IBS

3.1 Chapter Overview and Introduction

As has been communicated in section 1 of this thesis, currently, only IOP has been identified as a clinically significant modifiable risk factor for glaucoma[1030]. Broader understanding of risk factors for glaucoma should allow for the development of novel therapeutic agents.

There has been growing interest in the host-microbiome interactions that influence human health and disease[393, 394, 1031]. Animal research has shown that bacterial LPS can exacerbate RGC loss in glaucoma models[663]. More recently it was shown that microbiome interactions with the immune system may increase the propensity for immune mediated retinal damage in a mouse model of intraocular hypertension[245]. In human research it has been shown that glaucoma patients have higher oral microbiome loads than matched controls[663].

IBS is a chronic illness characterised by abdominal pain often in the context of bloating, and bowel dysfunction. There is accumulating evidence that IBS patients have abnormal microbiome[710, 711, 721]. IBS has been suggested as a risk factor for other neurodegenerative illnesses[570, 926]. Given the growing literature documenting the effects of the microbiome on the central nervous system effects, our group hypothesized that IBS may be associated with the glaucoma.

The present study utilised a case-control design to determine if a diagnosis of IBS is more common in adult physician-diagnosed glaucoma patients from the Australia and New Zealand Registry of Advanced Glaucoma (ANZRAG) compared with population-based controls.

The results offered limited scope to assess for co-variable information and therefore the presented work remains a proof-of-concept study to set the tone for the findings presented in Chapter 4.

3.2 Methods

Overview

The ANZRAG was compared to the Australian population-based Hunter Community Study (HCS) in a case-control study that assessed the odds of ROME-III defined IBS in people with physician diagnosed advanced glaucoma in comparison to controls from the general population.

Data Source

ANZRAG is voluntary registry of physician diagnosed and referred patients with advanced glaucoma that was established primarily for Genome-Wide Association Studies[1032].

The HCS is a population-based study of an aging population (age 55-85) in the Hunter Region of NSW, Australia. Aside from being slightly younger, the final cohort of 3207 participants, was statistically similar in demographics (gender, and marital status) to the Australian national population. A broader description of the cohort has been published previously[1033].

Identification of Irritable Bowel Syndrome

IBS was assessed in all participants by mailed surveys. The surveys comprised of a questionnaire containing the diagnostic criteria for IBS based on a modified ROME III questionnaire. Identical questionnaires (Appendix 2 and 3) were sent to the HCS and ANZRAG participants. Questionnaires sent to the HCS was included as part of a larger data collection sweep.

IBS, as defined by the ROME III criteria[1034], is a gastrointestinal syndrome described by abdominal pain or discomfort at least one day per week, as well as two of the following: 'pain relief on defecation', 'pain associated with alterations in the consistency of bowel motions', or 'pain associated with alterations of bowel frequency'. Defining IBS requires meeting a cut-off for frequency of associated symptoms. For the purposes of sensitivity analysis, two frequency boundaries were established, as described in Supplementary Methods (Appendix 4), to determine conventional and stringent definitions.

Identification of Glaucoma

The ANZRAG registry was the source of all glaucoma cases in the present investigation. The ANZRAG registry's selection criteria are based on the identification of significant central vision loss, due to glaucoma, identified on VF testing in at least one eye. Patients meeting the inclusion criteria, were only excluded from enrolment if their glaucoma was secondary to trauma, inflammation, aphakia, neovascularization or rubella. The subset of ANZRAG that had consented to ongoing contact (n=2,132) was the source of all glaucoma cases in this study.

Cases of suspected glaucoma were also identified in the HCS for the purposes of removing existing cases in the control population. In the HCS, glaucoma was identified by those who had, according to government prescription registries, used glaucoma eye drops, or who had undergone glaucoma surgery, and those who had disclosed a glaucoma diagnosis within the survey. HCS participants who met the criteria for glaucoma were excluded from this study.

Identification of Co-Variables

Gender and age at the time of answering the survey were the covariables available in this investigation.

Statistical Analysis

Cases from the ANZRAG cohort were matched to participants from the HCS in a ratio of 1:2, based on age group at the time of survey (in 5-year segments) and gender. Matching occurred between participants for whom all necessary data was available. The primary exposure of interest was IBS, identified using a modified ROME III questionnaire and coded as either yes or no. The primary outcome measure was glaucoma coded as either yes or no.

Statistical analyses were performed using univariable and multivariable logistic regression. Although matching based on age was performed using a range of 5 years, this was included in multivariate models as a continuous variable. Results were reported as Odds Ratios (OR) with 95% confidence intervals (CIs). $P<0.05$ was considered statistically significant.

3.3 Results

1021 participants responded to the ANZRAG survey, a response rate of 48.6%, however complete demographic data was only available on 803 participants. In the HCS, 2251 participants completed the survey (response rate of 67.8%). IBS, according to regular and stringent definitions, was identified in 451 and 243 participants, respectively, across both cohorts. 237 members of the HCS (10.5%) were identified with existing glaucoma and were excluded from the control group prior to matching. Prior to matching, the ANZRAG cohort was older and had a higher prevalence of IBS than the HCS cohort (Table 3.1).

Table 3.1: Description of data collected from ANZRAG and HCS

	ANZRAG (n=1021)	HCS (2251)	P value
Age	71.5*	67.7	**<0.001**
Gender (Male)	372 (46.3%)*	1060 (47.1%)	0.69
IBS - conventional	197 (19.9%)	254 (11.3%)	**<0.001**
IBS - stringent	103 (10.4%)	140 (6.2%)	**<0.001**

*n=803

Odds ratio's for IBS in ANZRAG glaucoma patients compared with HCS controls are displayed in Table 3.2. Multivariable analysis demonstrated that glaucoma patients were 1.78 (p<0.001, 95%CI 1.31-2.43) times more likely to have IBS, when using a stringent definition for IBS; and 1.93 (p<0.001, 95%CI 1.52-2.44) times more likely when using the regular definition of IBS.

Table 3.2: Odds ratios for identification of IBS in people with glaucoma compared to a matched cohort taken from the general population in Australia

	Univariable OR (95% CI)	Multivariable* OR (95%CI)
IBS - conventional	1.91 (1.54-2.38)	1.93 (1.52-2.44)
IBS - stringent	1.71 (1.54-2.38)	1.78 (1.31-2.43)

*Model adjusted by gender and age

Given that gender has such a strong correlation with IBS (p<0.001 for both IBS definitions in this cohort), the interaction between gender and glaucoma was assessed as a potential effect modifier. The interaction between gender and glaucoma is not significant in the regular definition of IBS (p=0.62), and when limited to men, this regular IBS remains

significantly associated with glaucoma (OR: 1.74, p<0.01, 95%CI 1.19-2.54). However, the interaction between gender and glaucoma is significant with regards to the stringent definition of IBS (p=0.044), and, when limited to men, strict IBS is no more common in men with glaucoma than without (OR 1.07, p=0.78 95%CI 0.62-1.86). When limited to women, the association for identifying IBS in women with glaucoma is strong for both definitions, with OR of 2.08 for regular IBS (p<0.001 95%CI 1.54-2.81) and OR of 2.36 for the stringent definition of IBS (p<0.001, 95%CI 1.62-3.45).

3.4 Discussion

The present investigation sought to determine if people with glaucoma in Australia are more likely to have IBS. In a case-control study established between a cohort of people with advanced glaucoma, and a population-based cohort of controls, glaucoma patients were almost twice as likely to also have a diagnosis of IBS.

To the author's knowledge, this association has not been explored in any great depth previously. Therefore, this finding should be confirmed with replication studies, and until this is done the findings should be interpreted with caution. Nevertheless, the present study offers some evidence that IBS, defined by the ROME-III criteria, is significantly more prevalent in a cohort of well-defined glaucoma patients as compared to a representative sample of the Australian population.

The theoretical basis of this study was the developing literature that has linked microbiome alterations to CNS physiology. Given IBS's relationship with disturbed microbiome[710, 711, 721], it was hypothesized that if these changes could alter CNS homeostasis[1031], IBS may be associated with glaucoma. Although the present study cannot comment on this relationship, the microbiome-CNS axis may be a relevant pathway responsible for the association seen. Alternatively, IBS has been thought of as a low-grade inflammatory illness; it has been linked to disturbed immune system function with some studies finding elevated cytokine concentrations and increases in small bowel derived T cells in the circulation of people with IBS[786, 787, 791]. Indeed there has also been some suggestion that inflammation may play a role in the pathogenesis of glaucoma, although these findings are typically local and do not include a systemic component[1035]. Further research will be required before a mechanism can be identified.

The primary limitation of this study is the limited opportunity for the assessment of confounding, as such these findings may be the result of residual confounding. The clearest risk factors for glaucoma are ocular biometry and there is no evidence to suggest that IBS would influence these. Beyond this, the largest risk factor for glaucoma is age[17], however the present study was well controlled for age both in design and in statistical analysis. Ethnicity was unable to be controlled for in the present study; black ethnicity is related to the risk of glaucoma[17], however ethnicity does not appear to have a significant relationship with IBS[823], and therefore this is unlikely to confound the effect, especially given the largely homogenous racial background of the aging Australian population. Aside from the pathologies of interest,

other pathologies were also unable to be accounted for in this study. Diabetes mellitus is a small but significant risk factor for glaucoma[111], and the relationship between Diabetes and IBS is unclear[828], although this is likely to be a weak confounder, future research should evaluate its role as a confounder in this relationship. Finally, one must consider if IBS is a more sophisticated expression of gastrointestinal disturbance associated with glaucoma medications, which do have some gastrointestinal effect through their limited systemic absorption and lacrimal drainage. Beyond these, based on an extensive review of the literature there are limited shared risk factors that are likely to confound this relationship.

The implications of this investigation primarily involve the direction of future research. In addition to the obvious replication and longitudinal studies that are required to determine the validity of these findings, ongoing work will be required to identify a mechanism for the biological relationship between these illnesses. Two potential mechanisms have been described above; further research will tell if these are relevant to glaucomatous pathology.

Chapter 4 – Adults with IBS are More Likely to Develop Glaucoma

4.1 Chapter Overview

In the previous chapter proof-of-concept data was presented that demonstrated that people with glaucoma are more likely to have IBS than members of the general population (matched by age and gender). This finding requires significant elaboration in the pursuit of establishing a link between IBS and glaucoma.

In the development of this research we searched for large cohort studies that allowed for investigation of both IBS and glaucoma with data available to researchers. We identified two large population cohorts where data on IBS has been collected at multiple timepoints with incidence data for glaucoma also available. These cohorts formed the basis for the manuscript that makes up the basis of the following chapter.

In this chapter, the relationship between IBS and incident glaucoma is assessed. The cohorts chosen allow for a broader assessment of confounding and indirect causation pathways than was available for the investigation in chapter 3. The results indicate that IBS may be a risk factor for glaucoma.

This chapter was a manuscript that had been prepared for submission to the journal Gut. A subsequently reformatted version of this work has been submitted to Annals of Internal Medicine. Its citation is as follows:

McPherson ZE, Sørensen HT, Horváth-Puhó E, Agar A, Coroneo MT, White A, Francis IC, Pasquale LR, Kang JH, Pettersson S, Talley NJ, McEvoy M. Irritable bowel syndrome and risk of glaucoma: an analysis of two independent population-based cohort studies.

The figures, tables and references in the following chapter have been renumbered in line with the formatting of this thesis.

4.2 Abstract

Objectives: Irritable bowel syndrome (IBS) is a chronic disorder associated with an abnormal gastrointestinal microbiome. Microbiome-host interactions affect the central nervous system. We hypothesized that IBS may be a risk factor for glaucoma, a neurodegenerative eye disease.

Design: Two prospective cohort studies in the United Kingdom and Denmark.

Study population: Participants in the 1958 UK Birth Cohort (UKBC; 9091 individuals) and patients with records in Danish National Patient Register (DNRP; 62,541 individuals with IBS, and 625,410 matched cohort members).

Methods: In the UKBC, participants were enrolled at birth and surveyed at specific ages (including ages 42 and 50) throughout life. Denmark's cohort contains records of diagnoses made by hospitals and procedures performed during hospital-based contacts, and prescription data from the national prescription database.

Main Outcome Measures: In the UKBC, incident glaucoma at age 50 was determined through comparison of survey responses at ages 42 and 50 years. In the DNPR glaucoma was assessed by: hospital diagnosis, glaucoma surgery, and initiation of glaucoma medications.

Results: In the UKBC, the odds ratio of developing glaucoma between ages 42 and 50 in persons with a persisting IBS diagnosis (at both ages 42 and 50) were increased [OR 5.84, 95% confidence interval (CI) 2.26-15.13]. People with an IBS diagnosis in the DNPR had a hazard ratio (HR) of 1.35 for developing physician-diagnosed glaucoma (95%CI 1.15-1.59), a HR of 1.34 for undergoing surgery for glaucoma (95%CI 1.04-1.74), and a HR of 1.19 for initiating use of glaucoma medication (95%CI 1.02-1.40).

Conclusions: IBS may be a risk factor for glaucoma.

Summary Box

What is already known:

- Glaucoma is a blinding illness with only one known clinically significant modifiable risk factor.
- IBS is associated with abnormal microbiome.
- Microbiome disturbance may impact on central nervous system physiology.

What this study adds:

- Irritable bowel syndrome may be a risk factor for glaucoma.

Clinical Impact:

- Recognizing this as a new risk factor may allow for greater surveillance of glaucoma (often asymptomatic until late in the disease process)
- This finding may allow for investigation of new therapeutic options for glaucoma.

4.3 Introduction

Glaucoma is a neurodegenerative disease affecting the optic nerve with a number of different phenotypic patterns[1036]. Globally it is a leading cause of irreversible blindness, with studies suggesting that approximately one in six persons with glaucoma will go blind during their lifetimes[1037]. Intraocular pressure is now the only known modifiable risk factor for glaucoma. Elucidation of novel risk factors is needed to inform clinical decision making regarding screening for glaucoma and also may provide insights into potential disease pathways, paving the way for development of neuroprotective interventions [1030].

Interactions between host and microbiome are known to influence human health[393, 394]. In one small study, bacterial loads in the oral microbiome of glaucoma patients were shown to be significantly higher than in healthy controls[663]. In animal models, a low dose of a bacterial toxin, lipopolysaccharide, was found to exacerbate glaucoma through activation of the immune system[663]. Recent work has demonstrated that the optic nerve degeneration can be driven by microbiome dependant auto-immune mechanisms[245].

Irritable bowel syndrome (IBS) is a chronic disorder characterized by abdominal pain, and bowel dysfunction. Evidence is accumulating that the gastrointestinal microbiome of IBS patients is abnormal compared to healthy populations[721, 724]. Furthermore, immune activation may occur, with circulating small intestinal homing T cells and cytokine release[791, 1038]. Importantly, the gut microbiome regulate both the concentration of neuroprotective neurotrophins[513, 516] and the activity of microglia[540] in the central nervous system (CNS). This suggests that dysbiosis of the gut microbiome could play a role in neurodegenerative disease. Alteration of the stool microbiome has been reported in Parkinson's disease and other neurodegenerative diseases[559, 588, 597]. It is possible that gut-mediated neuroprotection may extend to the retina. As well, this neuroprotection may be lost or diminished by changes in the gut microbiome, as observed in IBS.

This research aimed to examine whether there is an association between IBS and glaucoma. We hypothesized that adults diagnosed with IBS are at increased risk of developing glaucoma.

4.4 Methods

Overview

This study examined the risk of a glaucoma diagnosis in adults with and without an IBS diagnosis who participated in the United Kingdom-based 1958 Birth Cohort (UKBC) or who had records in the Danish National Patient Register (DNPR).

Data Sources

The 1958 Birth Cohort

The UKBC Birth Cohort study was initiated in 1958 with the enrolment of approximately 17,500 children born in the United Kingdom during a single week in 1958. All children delivered were eligible for enrolment and 98.7% of potential cohort members participated[1039]. Cohort members were followed up at ages 7, 11, 16, 23, 33, 42, and 50 years with broad surveys addressing demographics, health, and many other characteristics. During the years in which cohort members attended school, immigrant children born in the reference week were added to the sample.

Out of 16,091 possible respondents, 11,419 (71%) completed the survey at age 42. At age 50, 9,790 out of 15,806 (62%) responded. The cohort size at each wave was reduced due to mortality and international migration. However, the primary reason for attrition over time occurred when participants moved to a new address and did not respond to efforts to trace them. The largest drop in the response rate occurred after completion of schooling[1039]. The UKBC is described in detail in by Power and Elliott[1040].

The UK data service provides access to UKBC data for non-commercial use[1041-1044].

The Danish Cohort

The Danish National Patient Registry (DNPR) is a population-based registry that has recorded all hospital-based care provided to residents of Denmark since 1977. Its coverage expanded from inpatient care to include hospital outpatient clinic and emergency department care in 1995. The registry includes both primary and secondary diagnoses reported by treating hospitals. All records in the DNPR are identified by patients' unique civil registration number, which is assigned to all Danish citizens at birth and to all residents upon immigration. During the enrolment period for the present study (1 January 1995 to 30

November 2013) a cumulative population of 7,298,249 persons had records in the DNPR. The scope of the DNPR and associated methodology are explained in greater detail in the review by Schmidt *et al*[1045].

The current cohort study assessed the incidence of glaucoma in patients diagnosed with IBS during 1995-2013. This study prospectively included Danish residents with a hospital diagnosis of IBS (n=62,541) in the DNPR. Two comparison cohorts also were identified. The first included age- (year of birth) and gender-matched general population comparison cohort members drawn from the general population (10 cohort members per IBS patient, n=625,410). The second consisted of age- (year of birth) and gender-matched comparison cohort members who had a hospital diagnosis of cholelithiasis recorded in the DNPR during the enrolment year of the IBS patient (one cohort member per IBS patient, n=62,541).

Approval for use of DNPR data was granted by Aarhus University and Statistics Denmark.

Identification of Irritable Bowel Syndrome

In the 1958 British Birth Cohort study, IBS was assessed by self-report in surveys administered at ages 42, and then again at surveys administered at age 50 (See Supplementary Methods, Appendix 5, for full explanation). Participants who met the criteria for a case of IBS at or before the age of 42 and also at the age of 50 were considered to have 'persistent IBS'.

In the DNPR all patients with an inpatient or outpatient hospital clinic contact for IBS from 1 January 1995 to 30 November 2013 were enrolled in the study. IBS was classified based on *International Classification of Diseases, Eighth Revision* (ICD-8) codes up to 1993 and subsequently by *Tenth Revision* (ICD-10) codes (Supplementary Methods, Appendix 5). The date of the first entry of an IBS diagnosis code into the DNPR was defined as the IBS diagnosis date.

Identification of Glaucoma

In the 1958 British Birth Cohort, glaucoma was assessed by self-report in surveys administered at ages 16, 42, and 50 (Supplementary Methods, Appendix 5). As no cases of glaucoma were identified at age 16, it is unlikely that any cases of congenital glaucoma were

represented in the cohort. A participant was considered to have a case of 'incident glaucoma' if they met the criteria for glaucoma at age 50 but not at age 42.

In the Danish cohort, three definitions were used to identify glaucoma to reduce risk of misclassification: (1) physician diagnoses of Primary Open Angle Glaucoma made in discharge diagnoses or hospital outpatient clinics using ICD-8 and ICD-10 codes, as recorded in the DNPR; (2) surgical procedures interventions performed in hospitals, documented using the Nordic Classification of Surgical Procedures and (3) first-time redemption of a prescription for a medication used to treat glaucoma, as recorded in the Danish National Health Service Prescription Registry using the Anatomical Therapeutic Chemical classification (full coding is available in Supplementary Methods). As medication data became available in 2004, analyses that relied on redeemed prescriptions to identify glaucoma were restricted to 2004-2013.

Covariables

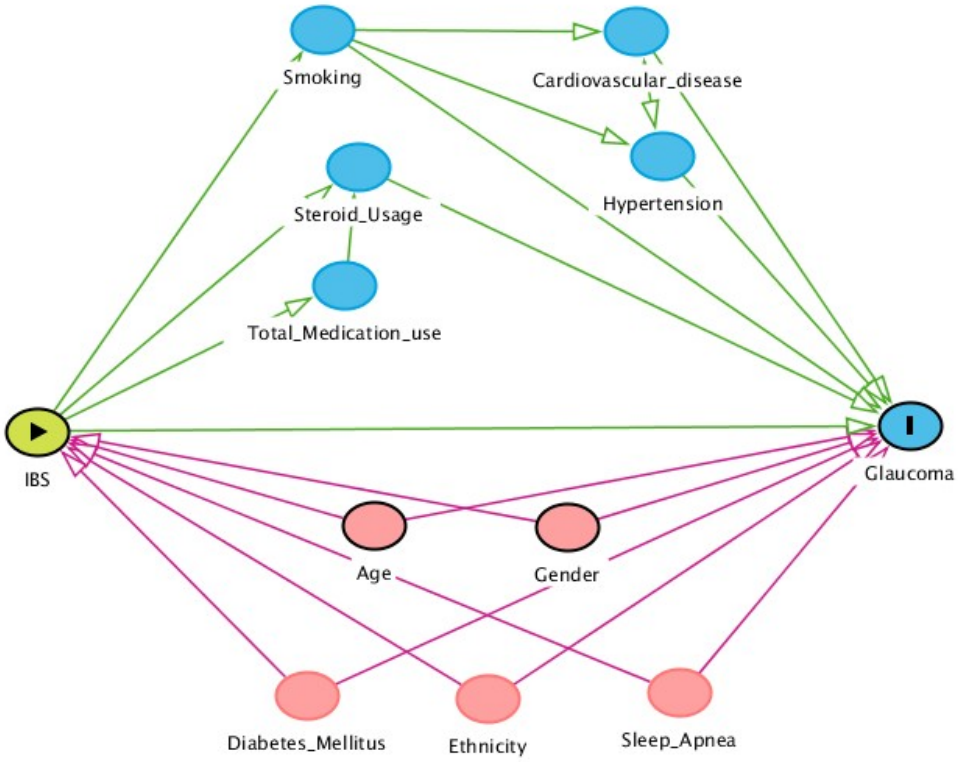
Potentially confounding covariables were identified from a literature review of risk factors associated both with IBS and glaucoma. A Directed Acyclic Graph (Figure 4.1) was used to assess covariates for potential to confound the association. Potentially confounding factors included age, ethnicity, gender, sleep apnoea and diabetes mellitus.

The covariables available in the UKBC analysis were age, gender, diabetes mellitus, ethnicity and smoking. As the study used a birth cohort population, age was already accounted for by the study design. Gender was recorded at time of study enrolment. Diabetes mellitus (coded as yes or no) was identified by self-report and was assessed at ages 7, 11, 16, 42 and 50 years. If participants indicated a diagnosis of diabetes at any time, they were considered to have the disease. Ethnicity was ascertained in the survey administered at age 42. Smoking was ascertained in the survey administered at age 50, when participants were asked if they currently smoked and if they had ever smoked.

The covariables available in the DNPR analysis were age, gender, diabetes mellitus, sleep apnoea, Chronic Obstructive Pulmonary Disease (COPD) and steroid usage. Age and gender were already accounted for by the study design. Data on diabetes mellitus, sleep apnoea and COPD were collected from diagnoses recorded in the DNPR (ICD-8 and ICD-10 codes), diabetes was also assessed identified with prescription data recorded in the Danish National Health Service Prescription Registry (ATC codes; see Supplementary Methods).

Figure 4.1: Directed Acyclic Graph identifying the potential causal structure linking Irritable Bowel Syndrome (IBS) and glaucoma

Literature review was performed to identify factors associated with both IBS and with Glaucoma, and causal paths were plotted. Factors with evidence of causing both IBS and glaucoma are plotted in red, factors with evidence of causation by IBS but that may cause glaucoma are plotted in blue. Causal pathways (direct and indirect) are plotted with green arrows and confounding pathways were plotted with red arrows. Factors associated with only IBS or glaucoma alone are not plotted. Created with daggity.net[1046]



Statistical Analyses

In the UKBC analysis, the primary exposures of interest were IBS at or before the age of 42, and a chronic IBS. The primary outcome measure was a diagnosis of incident glaucoma occurring between the ages of 42 and 50. Logistic regression models were used to assess the odds of a glaucoma diagnosis among persons with a diagnosis of IBS, compared to those without IBS. Bivariate logistic regression was used to examine the crude unadjusted effect, and multivariate logistic regression was used to assess the odds of glaucoma after adjusting for potential confounders.

Due to the small number of glaucoma cases, it was not possible to adjust for smoking history or ethnicity in multivariate models. To assess these covariates, we performed subgroup analyses using the multiply-adjusted logistic regression model. The first subgroup analysis was limited to white participants. As smoking is a variable that may lead to an indirect causal pathway (Figure 4.1), a second subgroup analysis was limited to non-smokers to exclude this indirect pathway.

Participants with missing data were removed in all analyses. Results were reported as prevalence odds ratios (ORs) with 95% confidence intervals (CIs). Statistical analyses were completed using STATA software version 15.1 (StataCorp LLC, TX).

The DNPR analysis examined the cumulative incidence of glaucoma in hospital diagnosed IBS patients and comparison cohorts. IBS patients were compared to two comparison cohorts. The first consisted of members of the general population matched 10:1 by gender and birth year, selected at random and enrolled in the same year as their referent case. The second consisted persons with records in the DPRP who were diagnosed with cholelithiasis prior to the index date, matched 1:1 by gender and birth year, and also selected at random. Cholelithiasis is a separate disease characterized by abdominal pain and not currently associated with glaucoma or IBS[1047], serving as a good negative comparison group. The matching was done without replacement. All participants diagnosed with glaucoma (by any definition) prior to their enrolment in the study were excluded from the analyses.

The DNPR cohort data was analysed using a Cox proportional hazards regression model. The index date was defined by the date that IBS was diagnosed in persons with IBS, or the date for which IBS was diagnosed in the referent case in participants from the comparison groups. The hazard ratios were adjusted for age, sex, and calendar period (by study design) and for diagnoses of diabetes and sleep apnoea prior to the index date. For glaucoma

described by medication data, models corrected for steroid usage are also presented to identify if this is a significant indirect causation pathway in this study (Figure 4.1). Unadjusted and adjusted model results are presented for each outcome definition of glaucoma. As a sensitivity analysis, a lagged analysis for each outcome also was performed. In this analysis patients with glaucoma diagnosed within 1 year of an IBS diagnosis were excluded.

A secondary analysis was performed with COPD taken as another potential confounder as COPD is a marker of smoking history. Data are presented with hazard ratios (HRs) and 95% confidence intervals (CIs).

The proportional hazard assumption was assessed and was not violated across the study period. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC).

Residual Confounding Assessment

As confounding could distort the association of interest, and as identification of potential confounders is difficult given the significant differences in the two pathologies, E values[1048] were calculated for the point estimates for required unmeasured confounding to explain the effect sizes seen. These were calculated with www.evalue-calculator.com[1049] for each of the effect sizes, of the multiple adjusted models seen in each of the two cohorts.

4.5 Results

1958 UK Birth Cohort

Over 11,000 participants in the UKBC responded to one of the surveys administered at ages 33, 42, and 50 years. All three surveys were completed by 9091 participants (52.2% of the original sample).

Within this study population, 778 (8.6%) participants reported IBS at or before the age of 42. Persistent IBS was identified in 162 (1.8%) participants. Incident glaucoma, between the ages of 42 and 50 years, was identified in 48 (0.5%) participants. Characteristics of the study sample are presented in Table 4.1.

The odds of developing glaucoma between the ages of 42 and 50 among UKBC members with a diagnosis of IBS at or before age 42 are presented in Table 4.2. In the unadjusted model, those with IBS at or before age 42 had more than twice the odds of receiving a diagnosis of glaucoma between ages 42 and 50 (OR 2.15, 95% CI 1.00-4.61), compared with those without IBS at or before age 42. The effect was marginally attenuated and not statistically significant in the multivariate model (OR 1.96, 95% CI 0.91-4.26).

Table 4.1: Descriptive data for members of the 1959 UK Birth Cohort (UKBC) in total and by IBS diagnosis at age 42

	IBS before/at age 42 (n=778)	No IBS before/at age 42 (n=8313)	Total (n=9091)
Gender (female)	563 (72.4%)	4,116 (49.5%)	4,679 (51.5%)
IBS at age 50	162 (20.8%)	177 (2.1%)	339 (3.7%)
Glaucoma at age 50 Incident glaucoma between ages 42 and 50	9 (1.2%) 8 (1%)	43 (0.5%) 40 (0.5%)	52 (0.6%) 48 (0.5%)
Diabetes mellitus	45 (5.8%)	365 (4.39%)	410 (4.5%)
Ethnicity -White -Asian (including Indian) -Black -Other (including mixed decent)	 766 (98.5%) 4 (0.5%) 3 (0.4%) 5 (0.6%)	 8123 (97.7%) 55 (0.7%) 38 (0.5%) 97 (1.2%)	 8889 (97.8%) 59 (0.6%) 41 (0.5%) 102 (1.1%)
Smoking History at age 50* -Never smoked -Ex-smoker -Smoker	 339 (42.6%) 266 (34.2%) 173 (22.2%)	 3920 (47.2%) 2558 (30.8%) 1814 (21.8%)	 4259 (46.9%) 2824 (31.1%) 1987 (21.9%)

*Smoking data were not available for 21 participants

The odds of developing glaucoma between ages 42 and 50 in cohort members with a diagnosis of persistent IBS (IBS at both age 42 and age 50) are presented in Table 4.2. In the unadjusted model, persons with persistent IBS had more than six times the odds of developing glaucoma (OR 6.58, 95% CI 2.57-16.84), compared with those without chronic IBS. This effect was marginally attenuated and remained statistically significant in the fully adjusted multivariate model (OR 5.84, 95% CI 2.26-15.13), and in multivariate analyses restricted to the white population (OR 5.97, 95% CI 2.29-15.51).

Smoking may be a source of indirect causation; therefore, subgroup analyses of the multiple adjusted models were performed to determine the smoking independent effect. When the analysis of the odds people with persisting IBS, for developing glaucoma between age 42 and 50, were limited to non-smokers, the elevated odds were not substantially altered (OR 6.73, 95% CI 2.31-19.59).

Table 4.2: Results from the UK Birth Cohort (UKBC) examining the associations between IBS and glaucoma

Association	Bivariate OR (95%CI)	Multiply-adjusted* OR	Multiply-adjusted* model restricted to:	
		OR (95% CI)	White population OR (95% CI)	Non-smokers OR (95% CI)
IBS before/at age 42, associated with incident glaucoma between ages 42 and 50	**2.15 (95%CI 1.00 - 4.61)**	1.96 (95%CI 0.91-4.26)	1.74 (95%CI 0.85-3.97)	2.07 (95%CI 0.84-5.06)
IBS at/before age 42 AND at age 50, with incident glaucoma between ages 42 and 50.	**6.58 (95%CI 2.57-16.84)**	**5.84 (95% CI 2.26-15.13**	**5.97 (95%CI 2.29-15.51)**	**6.73 (95%CI 2.31-19.59)**

All data are presented as odds ratios with 95% confidence intervals. Associations that are significant ($p<0.05$) are bolded. *Adjusted for gender and comorbid diabetes mellitus

The Danish Cohort Analysis

Irritable Bowel Syndrome (IBS) was identified in 62,541 persons with records in the DNPR (0.85% of all persons in the Registry). Characteristics of the IBS and comparison cohort groups are presented in Tables 4.3 and 4.4.

During 499,761 person-years of follow up of IBS patients, and 5,007,551 person-years of follow up of matched comparison cohort members, 176 IBS patients and 1334 were found to have physician-diagnosed glaucoma. Glaucoma surgeries were identified for 69 IBS patients and 513 members of the comparison cohort. During 135,530 person-years of follow up of IBS patients and 1,351,772 person-years of follow up the matched comparison cohort in the 2005-2013 period (when medication data were available), 179 IBS patients and 1495 population cohort members were identified as initiating glaucoma medications.

Table 4.3: Characteristics of IBS patients identified from the Danish National Patient Register (DNPR) and their matched controls

	IBS cohort (n=62,541)	Matched general population cohort (n=625,410)	Cholelithiasis cohort (hospital comparison cohort) (n=62,540)
Gender (female)	43,000 (68.8%)	430,000 (68.8%)	43,000 (68.8%)
Glaucoma - hospital diagnosis - Surgery	 176 (0.3%) 69 (0.1%)	 1334 (0.2%) 513 (0.1%)	 146 (0.2%) 45 (0.1%)
Diabetes mellitus	2,510 (4.0%)	19,294 (3.1%)	3,797 (6.1%)
Sleep apnoea	415 (0.7%)	2003 (0.3%)	371 (0.6%)
Age at cohort enrolment - <60 - 60-79 - 70-79 - 80+	 46,584 (74.5%) 8,565 (13.7%) 5,163 (8.3%) 2,229 (3.6%)	 465,720 (74.5%) 85,925 (13.7%) 51,427 (8.2%) 22,338 (3.6%)	 46,588 (74.5%) 8,563 (74.5%) 5,145 (8.2%) 2,244 (3.6%)
Year of cohort enrolment - 1995-1999 - 2000-2004 - 2005-2009 - 2010-2013	 13,058 (20.9%) 17,192 (27.5%) 17,770 (28.4%) 14,521 (23.2%)	 130,580 (20.9%) 171,920 (27.5%) 177,700 (28.4%) 145,210 (23.2%)	 13,058 (20.9%) 17,191 (27.5%) 17,770 (28.4%) 14,521 (23.2%)
Median years of follow-up (interquartile range)	7.58 (3.53-11.92)	7.61 (3.53-11.97)	7.58 (3.54-11.91)

After adjustment for potential confounders, a hospital diagnosis of glaucoma was more frequent in participants with IBS than in population cohort (HR: 1.35, 95% CI 1.15-1.59; Figure 4.2). IBS patients were at similarly increased risk for glaucoma surgery (HR: 1.34, 95% CI 1.04-1.74) and glaucoma medication use (HR: 1.19, 95% CI 1.02-1.40). These results remained robust in our lagged analysis, which excluded patients whose glaucoma was diagnosed within 1 year of their IBS diagnosis were excluded (Table 4.5).

Although steroids are not a treatment for IBS, people with IBS more commonly used steroids in this cohort (Table 4.4). To identify the steroid independent effect of IBS on glaucoma, the model presented in Table 4.5 was further adjusted for steroid usage. Despite adjustment there was minimal alteration to the effect size seen between IBS and glaucoma as defined by medication usage, with HR of 1.18 (95% CI 1.01-1.43). Lagged analysis was similarly unaffected; HR 1.21 (95%CI 1.02-1.43). To identify an effect size of 1.35, survival analysis with a 1:10 ratio of exposed to unexposed requires 1054 events[1050], unfortunately amongst the cohort limited to 2004-2013 only 80 surgery cases and 181 physician diagnoses were identified, and therefore these analyses could not be performed.

When the IBS cohort was compared to the comparison cohort diagnosed with cholelithiasis (Table 4.6), the association between IBS and physician-diagnosed glaucoma was attenuated (HR: 1.25 95% CI 0.98-1.59). However, the associations with glaucoma defined by surgical (HR: 1.68, 95% CI 1.10-2.58) and medical interventions (HR: 1.28, 95% CI 1.01-1.63) remained robust. When the medication definition is controlled for steroid use, the effect size is completely unchanged (HR: 1.28, 95%CI 1.01-163).

Although, COPD is not specifically a confounding factor (Figure 4.1), as smoking is strongly associated with COPD, an additional analysis was performed controlling for COPD with the aim of assessing the potential of smoking to be involved in the causal pathway. Although people with IBS were more likely to also have COPD (Supplementary table 4.3, Appendix 5), the results adjusted for COPD were not substantially different and are presented in Supplementary Tables 4.4 and 4.5 (Appendix 5).

Table 4.4: Characteristics of IBS patients Identified from Danish National Patient Register (DNPR) and their matched comparison cohort during 2004 -2013, when medication data were available

	IBS cohort (n=32,291)	Matched general population cohort (n=322,910)	Cholelithiasis cohort (hospital comparison cohort) (n=32,291)
Gender (female)	22,247 (68.9%)	222,470 (68.9%)	22,247 (68.9%)
Glaucoma - Medication initiation	179 (0.6%)	1495 (0.5%)	150 (0.5%)
Diabetes mellitus	1,752 (5.4%)	13,092 (4.1%)	2,527 (7.8%)
Sleep apnoea	348 (1.1%)	1681 (0.5%)	311 (1.0%)
Steroids usage (Redeemed prescription)	5025 (15.5%)	30,931 (9.6%)	4,174 (12.9%)
Age at cohort enrolment - <60 - 60-79 - 70-79 - 80+	 24,417 (75.6%) 4,505 (14.0%) 2,353 (7.3%) 1,016 (3.1%)	 244,176 (75.6%) 45,142 (14.0%) 23,344 (7.2%) 10,248 (3.2%)	 24,419 (75.6%) 4,513 (14.0%) 2,335 (7.2%) 1,024 (3.2%)
Median years of follow-up (interquartile range)	4.11 (1.88-6.50)	4.09 (1.86-6.49)	4.11 (1.89-6.50)

Table 4.5: Results from the Danish National Patient Register (DNPR): risk of glaucoma in persons with IBS compared to the general population

Glaucoma Definition	Cumulative Incidence Risk		Unadjusted hazard ratio	Adjusted hazard ratio*
	General population cohort	IBS patients		
Physician diagnosis	0.47 (0.43-0.50)	0.72(0.53-0.95)	**1.36 (1.16-1.59)**	**1.35 (1.15-1.59)**
Physician diagnosis (lagged)	0.45 (0.42 - 0.49)	0.70 (0.51 - 0.93)	**1.32 (1.11–1.56)**	**1.31 (1.10–1.55)**
Glaucoma surgery	0.24 (0.20-0.28)	0.28 (0.20-0.38)	**1.37 (1.06-177)**	**1.34 (1.04-1.74)**
Glaucoma surgery (lagged)	0.24 (0.20 - 0.28)	0.27 (0.19 - 0.37)	**1.33 (1.02–1.73)**	**1.31 (1.01–1.71)**
Glaucoma medication initiation	1.01 (0.94-1.09)	1.11 (0.94-1.30)	**1.21 (1.03-1.41)**	**1.19 (1.02-1.40)**
Glaucoma medication initiation (lagged)	0.94 (0.87 - 1.02)	1.04 (0.87 - 1.23)	**1.23 (1.04–1.45)**	**1.22 (1.03–1.44)**

Data with 95% confidence intervals are presented for both the complete analysis and for the 1-year lagged sensitivity analysis. *Adjusted for diabetes mellitus and sleep apnoea diagnoses

Figure 4.2: Cumulative incidence curves of glaucoma, identified by A: hospital diagnosis, B: glaucoma surgery, and C: glaucoma medication initiation, in persons with IBS compared to the general population

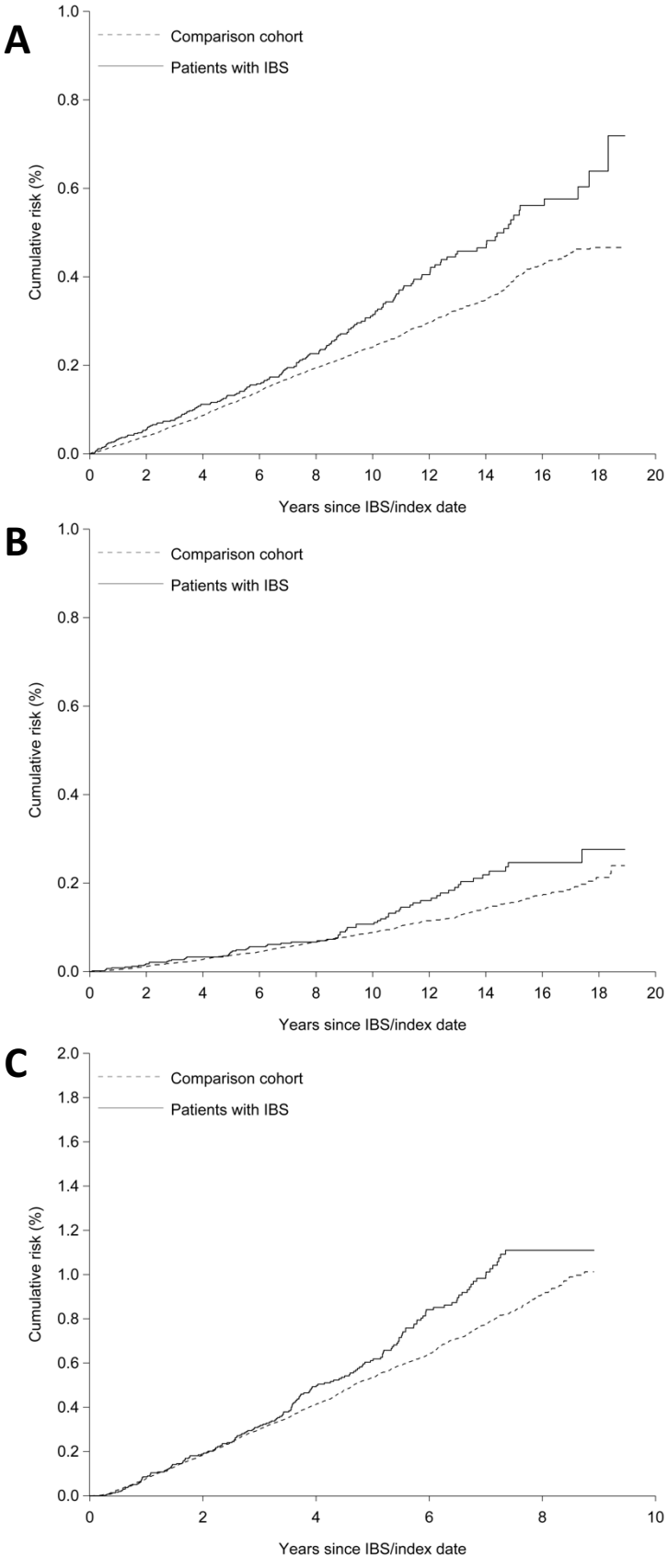


Table 4.6: Results from the Danish National Patient Register (DNPR): risk of glaucoma in persons with IBS compared to those with cholelithiasis

Glaucoma definition	Cumulative Incidence risk		Unadjusted hazard ratio	Adjusted hazard ratio
	Cholelithiasis cohort	IBS cohort		
Physician diagnosis	0.53 (0.43 - 0.66)	0.72 (0.53- 0.95)	1.24 (0.97– 1.57)	1.25 (0.98– 1.59)
Glaucoma surgery	0.18 (0.12 - 0.26)	0.28 (0.20- 0.38)	**1.59 (1.06– 2.41)**	**1.68 (1.10– 2.58)**
Glaucoma medication initiation	1.11 (0.86 - 1.40)	1.11 (0.94- 1.30)	**1.26 (1.00– 1.59)**	**1.28 (1.01– 1.63)**

Data are presented with 95% confidence intervals.

Residual Confounding Analysis

Effect sizes for required residual confounding to account for the effect sizes seen (E-values) were calculated for each adjusted model presented.

In the UKBC, the E-value for the association between persistent glaucoma from age 42 to age 50 and incident glaucoma in that time period was 11.16.

In the DNPR, E-values for the analyses compared to the population sourced cohort ranged from 1.67 for the medication definition to 2.01 and 2.04 for the surgery and physician diagnosis definitions. Compared to the cholelithiasis controls E values ranged from 1.81 and 1.88 for the physician diagnosis and medication definition to 2.75 for the surgical definition.

4.6 Discussion

This study examined whether IBS was a risk factor for glaucoma in two large European cohort studies. In our analyses of data from two large long-term prospective studies, a diagnosis of IBS was associated with an increased risk of glaucoma.

To the best of our knowledge, the association between gastrointestinal health and glaucoma has not been explored previously. Our findings thus should be interpreted with caution and confirmed with other studies. Further research is needed to exclude residual confounding.

Two Taiwanese studies have suggested a link between IBS and both Parkinson's Disease[926] and Alzheimer's Disease[570]. As glaucoma and these two conditions each have different aetiologies and natural histories, it is possible that IBS may have a negative impact on CNS homeostasis, particularly for neurodegenerative diseases. We suggest that the association between IBS and glaucoma could be due to two potential mechanisms: host-microbiome interactions or immune system dysregulation.

IBS appears to be associated with alterations in the microbiome (Pittayanon *et al*. 2019[724] for review). Although it is difficult to establish causal direction in the link between microbiome disturbance and IBS, the microbiome alterations associated with IBS may be one potential cause of the elevated risk of glaucoma in IBS patients. In support of this hypothesis, recent animal models have also suggested that the microbiome may play a role in glaucoma pathophysiology through a T cell mediated effect[245]. There are also multiple reports of reductions in the neurotrophin brain-derived neurotrophic factor (BDNF), an important neuroprotective peptide for the optic nerve[210, 343], in various CNS regions in germ-free[513, 515] and dysbiosis mouse models[516]. Disruptions in the microbiome also have been found to alter the blood-brain barrier and microglial function. (Lee *et al*. 2016[1031] for review).

The second potential mechanism that may explain the association between IBS and glaucoma involves alterations in the immune system. Although IBS has traditionally been considered a brain-gut disorder with normal gut pathology, there is increasing evidence of altered immune homeostasis. [786, 787] This may play a role in glaucoma pathology. There is some indication that IBS patients may have elevated inflammatory markers such as TNF-α, IL-6, and IL-8[787]. Moreover, increased amounts of circulating $\alpha4\beta7$ integrin (small bowel homing) T cells have been identified, implicating small intestinal inflammation in IBS [791, 1038].

There is a general consensus that low-grade inflammation may play a role in some IBS cases,[1051] presenting another potential mechanism that could link IBS and glaucoma. A controversial discussion has arisen regarding the possibility that low-grade inflammation plays a role in the pathogenesis of glaucoma[1035]. Although there has been some suggestion that certain inflammatory cytokines such as IL-6 may be elevated in the serum of people with glaucoma[1052], these cytokines have been shown to be lower in the aqueous humour of glaucoma patients[204] and even may even play an important protective role in axonal damage[207, 1053].

The current research has several important strengths. We were able to replicate our findings in two independent cohorts in different countries. In the UKBC, both the cross-sectional study and longitudinal analyses had similar findings. Consistent with a dose-response relation, 'chronic IBS' increased the effect of IBS on the risk of glaucoma compared to the effect when IBS was ascertained at only one time point. Similarly, in the DNPR-based study, IBS increased the hazard of glaucoma, and this was reiterated with multiple outcome definitions. The DNPR analysis also made use of a hospital comparison cohort, cholelithiasis, another disorder presenting with abdominal pain, to address concerns regarding biases that may occur in reporting illnesses to national registries. The analysis produced consistent results.

There are some weaknesses to note in this investigation. The UKBC provided limited time points to capture IBS and glaucoma data. Similarly, while the DNPR provided dates of each hospital diagnosis, allowing for a clearer understanding of the timeline, the left-truncation problem that occurs with hospital-based registries will have occurred here also and therefore future studies are warranted to understanding the timeline of this association. The UKBC study is likely to benefit from greater surveillance and lower incidence of false negatives as all participants were asked to report detailed medical histories contrasting with the DNPR's use of registry data. This shortcoming is best illustrated by the low prevalence of IBS in the DNPR (0.85%), which is considerably lower than that reported in the UKBC and other studies[706]. This low prevalence is likely due to absence of data from general practitioners, who are largely responsible for the care of these patients, in the DNPR.

Although the majority of glaucoma patients have primary open-angle glaucoma[1036], we were unable to determine the subtypes of glaucoma affecting the IBS patients that we identified in the UKBC. In the DNPR, although we limited physician-diagnosed glaucoma to

Primary Open Angle Glaucoma, the surgical and medication definitions are unable to be limited by subtype. Future research is needed to examine whether IBS is associated with particular glaucoma phenotypes.

We also were unable to categorize IBS into subtypes and thus could not ascertain if associations applied to all IBS subtypes or only to one. If the microbiome is responsible for the effects seen, future investigation of this association should attempt to dissect which IBS subtypes primarily contribute to this finding, as the microbiome may differ between IBS subtypes[724].

Known risk factors for glaucoma and IBS have relatively limited overlap, minimizing the potential for confounding. While gender and age were clear confounders of the association, the other covariables did not appear to confound the effect to a significant degree, with effect sizes remaining essentially unchanged even in multiple regression analyses. Smoking and Steroid Use are also potential indirect causal pathways that could explain a link between IBS and glaucoma. In a model excluding smokers in the UKBC it was shown that smoking had limited effect on the effect sizes seen, similarly adjusting for COPD (a marker for smoking history) did not confound the effect seen in the DNPR. When steroid usage was adjusted for in the DNPR, minimal alteration to affect sizes was seen, suggesting that steroids are not an important indirect causal between IBS and glaucoma (by medication definition) in this cohort. Racial background, steroid usage and smoking history were difficult to evaluate fully. Subgroup analysis of the UKBC, within the white population, demonstrated no significant impact on the results seen. This combined with the fact that the Danish population is racially homogenous, our results may be applied to white populations reliably. Although future prospective studies are needed to address such issues, it is unlikely that the absence of data on race in our analyses contributed significantly to residual confounding.

Future research is needed on management of glaucoma patients with IBS. Careful investigation of the role of gut microbiome dysbiosis and immune system dysregulation in glaucoma's pathogenesis may lead to new therapeutic options. It is also important to investigate whether IBS patients are at elevated risk for developing other neurodegenerative illnesses[570, 926].

Chapter 5 – Oral Health May Be Associated with Glaucoma in Adult Men

5.1 Chapter Overview

Although IBS may be the most promising condition to act as a pathomarker for dysbiosis in epidemiological research, there are also other options for illnesses that may demonstrate altered microbiome. As has been described in Chapter 1.5.1 the oral health also presents an opportunity to infer information about the microbiome.

In summary, the results suggested that oral health measures (tooth loss, and tooth loss in the context of periodontitis are only a significant risk factor for glaucoma within two years of their occurrence. These findings may implicate the transient bacteraemia associated with tooth loss as a catalyst for glaucoma however the findings are not as strong as the IBS findings described in the previous chapters. Indeed, as the abstract and discussion both state, more research is required, and for this reason a great deal of care should be taken in clinical interpretation of the findings seen here.

The article that follows was published in the journal Ophthalmology. I was a contributing author involved in data interpretation, and manuscript preparation. The paper was submitted 15th March 2016, revised 16th June 2016, and accepted on 11th July 2016. The citation for this paper is as follows:

Pasquale LR, Hyman L, Wiggs JL, Rosner BA, Joshipura K, McEvoy M, McPherson ZE, Danias J, Kang JH. Prospective study of oral health and risk of primary open-angle glaucoma in men: data from the Health Professionals Follow-up Study. Ophthalmology 2016:123(11):2318-2327

Permission to reproduce this paper here may be found at Appendix 6. The tables have been renumbered in line with the formatting of this thesis.

5.2 Abstract

Purpose: Tooth loss or periodontal disease is associated with systemic endothelial dysfunction that is implicated in primary open-angle glaucoma (POAG). The relationship between oral health and POAG has received limited attention. Thus, we evaluated the association between dental history and risk of POAG and POAG subtypes.

Design: Prospective cohort study

Participants: Health Professionals Follow-up Study participants (40,536 men) followed biennially from 1986 to 2012. At each 2-year risk period, eligible participants were 40+ years old, free of POAG, and reported eye examinations.

Methods: Using validated questions, we updated participants' status on number of natural teeth, teeth lost, periodontal disease with bone loss and root canal treatments.

Main Outcome Measures: During follow-up, 485 incident cases of POAG were confirmed with medical records and classified into subtypes defined by intraocular pressure (IOP) (≥ or < 22 mm Hg) or by visual field (VF) loss pattern at diagnosis (peripheral loss only or early paracentral loss). Multivariable relative risks (MVRR) and 95% confidence intervals (CIs) were estimated.

Results: Number of natural teeth, periodontal disease or root canal treatment were not associated with POAG. However, compared to no report of tooth loss, a report of losing teeth within the past 2 years was associated with a 1.45 fold increased risk of POAG (95% CI=1.06, 1.97); in particular, a report within the past 2 years of both losing teeth and having a diagnosis of periodontal disease was associated with 1.85 fold increased risk of POAG (95% CI=1.07, 3.18). The associations with recent tooth loss was not significantly different for the POAG subtypes (p for heterogeneity ≥ 0.36), although associations were strongest in relation to the POAG subtypes with IOP < 22 mm Hg (MVRR = 1.93, 95% CI=1.09, 3.43) and with early paracentral VF loss (MVRR = 2.27, 95% CI=1.32, 3.88).

Conclusion: While the number of natural teeth was not associated with risk of POAG, recent tooth loss was associated with an increased risk of POAG. Because these findings may be due to chance, they need confirmation in larger studies.

5.3 Introduction

Oral infections, leading to tooth loss or periodontal disease, have been related to a multitude of systemic diseases, such as diabetes, cardiovascular disease, rheumatoid arthritis, certain cancers and neurodegenerative diseases.[1054-1059] There are several mechanisms underlying the association with systemic illnesses, as have been previously reviewed and summarized.[1054, 1055] Periodontitis, a common bacteria-induced oral inflammatory condition that destabilizes the tooth structural support apparatus, can produce a transient bacteremia, which may lead to systemic endothelial dysfunction and chronic inflammatory responses in various extra-oral tissues.[1060-1062] Second, inflammatory markers generated from the affected periodontal tissue can also travel via bloodstream to reach other tissue beds. For example, in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, there is growing evidence that peripheral inflammation exacerbates the development of neuronal cell loss.[1056, 1057] The third mechanism is the immune response to the bacteria, which involves the generation of antibodies to bacteria and their toxins, which may have off-target effects in extra-oral tissues (e.g., cross-reactive antibodies that contribute to atherosclerosis).[1063]

Primary open-angle glaucoma (POAG) is a leading cause of blindness worldwide and is a chronic disease characterized by neurodegeneration of RGCs and their axons. In a clinic-based case-control study among African-Americans,[663] compared to 45 controls, 58 glaucoma cases showed significantly higher oral bacterial loads and significantly fewer teeth, especially in older persons.[1064] The same research group[663] found that when glaucoma animal models were administered low-dose bacterial toxins, glaucomatous neurodegeneration ensued and was accompanied by microglial activation, upregulation of the complement system and toll-like receptor 4 signaling activity in the optic nerve. These results suggested that oral infections, particularly those that can lead to periodontal disease, may have systemic effects that can contribute to POAG.

We hypothesized that the vascular bed in the base of the tooth may be a conduit for inflammatory cytokines and microbes to access the systemic circulation and through that, the optic nerve head capillaries, leading to endothelial cell dysfunction that would compromise oxygen supply to the optic nerve axons. Periodontal disease is associated with impaired flow-mediated vasodilation; in addition, treatment of periodontal disease improves flow-mediated vasodilation.[1060-1062] Similarly, POAG has been associated with impaired flow-mediated vasodilation, and several studies have reported on genetic and environmental exposures

related to endothelial cell function in association with the early paracentral visual field loss subtype of POAG.[139, 1065-1067]

To further test the possible link between oral infections and POAG at the population level, we prospectively evaluated self-reported comprehensive analysis of oral health and risk of POAG and POAG subtypes using data from 40,536 men in the Health Professionals Follow-up Study participants followed for 25+ years.

5.4 Methods

Study population

The Health Professionals Follow-up Study (HPFS)[1068] is an ongoing cohort study initiated in 1986 when 51,529 U.S. male health professionals (dentists, veterinarians, pharmacists, optometrists, osteopathic physicians or podiatrists), aged 40-75 years responded to a mailed health questionnaire. In the HPFS, participants are followed every two years with questionnaires that ask about newly diagnosed diseases such as periodontitis and glaucoma as well as other health and lifestyle factors. The follow-up rate for the HPFS cohort is greater than 85%. This work was HIPAA-compliant, and the described research adhered to the tenets of the Declaration of Helsinki. The Human Research Committees of Brigham & Women's Hospital, Massachusetts Eye and Ear Infirmary and the Harvard School of Public Health approved this study. The Human Research Committee regarded participants' return of completed questionnaire(s) as implied informed consent.

Ascertainment of Primary Open-Angle Glaucoma (POAG) Cases and Subtype Classification

We included 485 confirmed incident cases of primary open-angle glaucoma. Glaucoma case ascertainment occurred every two years; in questionnaires, participants were asked about eye exams and physician-diagnoses of glaucoma. For participants who reported a diagnosis of glaucoma, we sought permission to contact their eye care providers. Eye care providers were asked to send all visual field (VF) tests as well as medical records that established the diagnosis or a completed glaucoma questionnaire that asked about maximal intraocular pressure (IOP), status of the filtration apparatus, optic nerve structural information, ophthalmic surgery, and VF loss. Finally, records were reviewed by a glaucoma specialist (LRP), masked to participants' dental history, to confirm POAG cases using standardized criteria.

For the majority of POAG cases (>70% of cases), the following criteria were met: (1) gonioscopy showed that the filtration angle was not occludable in either eye, (2) slit lamp biomicroscopy showed no evidence in either eye of pigment dispersion syndrome, uveitis, exfoliation syndrome, trauma, or rubeosis, and (3) at least 2 reliable tests had to demonstrate reproducible VF defects consistent with POAG. For the remaining POAG cases, the slit lamp exam and VF criteria were met, but documentation of pupil dilation without subsequent

adverse events was considered as evidence for non-occludable angles. For VF defects, we did not require a specific type of perimetry; however, full static threshold testing was documented in 95%, and kinetic VFs in <1%. For static threshold or suprathreshold tests, we used the following reliability definitions: fixation loss ≤ 33%, false positive rate ≤ 20% and false negative rate ≤ 20%. For kinetic VFs, a VF test was considered reliable unless the examiner noted test circumstances to the contrary.

New glaucoma diagnoses were self-reported by 4,239 HPFS participants. These were confirmed as various types of glaucoma or glaucoma suspect in 52%: potential POAG with VF loss (25%), only elevated IOP or optic disc cupping (15%), and other types of glaucoma/glaucoma suspect (12%). The remaining (48%) were unconfirmed, as participants (16%), or eye care providers (6%) were unreachable, participants denied permission for record review (9%), participants indicated the report was erroneous (15%) or eye care providers refuted the glaucoma diagnosis (2%). Among the 25% classified as potential POAG with VF loss, we included only the POAG cases that met our case definition (485 cases); other confirmed and unconfirmed self-reports were censored in the analyses as of the diagnosis date.

For secondary analyses, we classified cases into subtypes by IOP and by VF loss pattern at diagnosis. We defined subtypes of "high-tension" (n=341) and "normal-tension" POAG (n=144) as those with maximum untreated IOP > or ≤ 21 mm Hg, respectively. We defined subtypes by VF loss pattern: those with peripheral VF loss only (n=260) or early paracentral VF loss (n=147) or undetermined VF loss (n=78) with a method previously described.[1069] For POAG with peripheral VF loss only, nasal step, temporal wedge or Bjerrum scotoma was present with no paracentral loss. For POAG with early paracentral loss, there was 1) paracentral loss only or 2) paracentral loss with VF loss in the Bjerrum area and/or nasal step area in the same hemifield, but without any temporal wedge loss. We included the latter paracentral group because cases with only paracentral loss were uncommon (~21%) and cases with clear paracentral loss frequently also showed peripheral loss. Cases (n=78) with undetermined VF loss (i.e., VF loss in the paracentral and any temporal wedge region in the same eye or paracentral in one hemifield with peripheral loss only in the other hemifield) were censored in the analyses as of the diagnosis date.

Ascertainment of Oral Health

For determining the number of teeth and number of teeth lost, in 1986, we asked about the number of natural teeth, and in the follow-up questionnaires, we asked about any tooth loss during the previous 2 years. In a validation study of a general population sample, self-reported number of teeth was highly correlated with the actual number of teeth on clinical assessment (r=0.97).[1070]

To ascertain periodontal disease history, in 1986, we asked about any periodontal disease with bone loss, and every two years, we asked about any new diagnoses of periodontal disease with bone loss. In the HPFS, we validated this question among dentist participants[1071] and other study participants[1072] by obtaining radiographs from individuals with and without a self-reported history of periodontal disease. Radiographs were evaluated for bone loss in 32 sites of all posterior teeth present except for the third molars by dentists who were masked to participants' self-report. Bone loss assessed from the radiographs was used as the standard measure of cumulative periodontal disease. We observed overall high validity of positive responses: in dentist participants (n=140), the positive predictive value was 0.76 and the negative predictive value was 0.74;[1071] in non-dentist participants (n=212), the positive predictive value was 0.80 and the negative predictive value was 0.68.[1072]

Analysis Study Population

We excluded at baseline (=1986) the following HPFS participants, respectively: 1) 1,596 who did not respond to baseline SFFQs or had outlying total caloric intakes as one of the original aims was to study diet and glaucoma (fewer than 70 out of 131 items blank in the SFFQ, with a total caloric intake <800 or >4200 kcal/day), 2) 1,927 with prevalent cancers excluding nonmelanoma skin cancer, as cancer diagnoses could alter many health behaviors, 3) 1,036 with prevalent glaucoma, 4) 956 lost to follow-up or <2 years of baseline, 5) 3,273 who never reported an eye exam during follow-up and 6) 18 who were missing information on dental history at baseline. After these exclusions, 42,723 were eligible; however, at the beginning of each 2-year risk period, we applied additional provisional exclusions for age and eye exam status. For example, for the 1986-'88 risk period, 29,673 contributed person-time after we provisionally excluded participants (n=13,050) who were age<40 years and reported no eye exam. In later periods, those provisionally excluded were allowed in analyses if they

met eligibility criteria during follow-up. Thus, over the study period, 40,536 ever contributed person-time.

Statistical Analysis

Our main exposures of interest were number of teeth, diagnosis of periodontal disease, number of teeth lost (from 1988, when first asked, to 2012) and number of teeth with root canal treatment (from 1996, when first asked, to 2012), which were updated during follow-up with repeated questionnaire information. To reduce misclassification of updated number of teeth, if a participant did not return a questionnaire, then we imputed the value using the updated number of teeth as of the immediately prior questionnaire; if a response was missing for two questionnaire cycles in a row, then the participant was censored at that point in the analyses of number of teeth and number of teeth lost.

Our main outcome of interest was all POAG. We calculated incidence rates of POAG by dividing the incident cases by person-years accrued for each category. For age-adjusted analyses, we conducted Cox proportional hazards analysis stratified by updated age in months and the specific 2-year period at risk,[1073] derived the multivariable relative risks (MVRRs) and 95% confidence intervals (CIs). For multivariable analyses, we ran similar Cox models simultaneously controlling for potential glaucoma risk factors that were time-varying. We conducted tests for trend by evaluating the significance of a variable representing category midpoint values. Similar approaches were taken to evaluate POAG subtypes.

Potential covariates were updated biennially using all information from baseline: glaucoma family history, African ancestry, Asian ancestry, body mass index (BMI; 22-23, 24-25, 26-27, 28-29, 30+ kg/m^2), pack-years of smoking (1-9, 10-19, 20-29, 30+ pack-years), hypertension, diabetes, physical activity (quartiles of MET [metabolic equivalent]-hours/week), alcohol consumption (g/day) and caffeine intake (mg/day), updated number of eye exams reported during follow-up, self-reported history of cataract diagnosis or extraction, age-related macular degeneration, hypertension, diabetes, and recent report of physical examination (for health maintenance, for medical concerns or no report of a physical exam).

Secondary Analyses

We performed several secondary analyses. We separately analyzed the risks of developing POAG stratified by: high- (HTG) and normal-tension POAG (NTG) using the highest known IOP, and by pattern of VF loss: POAG with peripheral VF loss only (Peri-POAG) and early paracentral loss (Para-POAG). For testing whether the associations with one POAG subtype are different from those with another subtype, we used the Lunn-McNeil approach[1074] to derive the p for heterogeneity [p-het]. Also, we conducted sensitivity analyses, where, for each dental history variable, we additionally adjusted for other dental history related variables as appropriate: updated number of teeth (continuous), periodontal disease history (none, diagnosis in past >2 years prior, diagnosis within 2 years), and updated number of teeth lost (0, 1, 2+). To evaluate detection bias, i.e., whether better screening practices leads to both greater dental care and diagnoses of periodontal disease as well as diagnoses of glaucoma, we repeated analyses among those who were 65 years or older (who tend to get more frequent health care overall). We also repeated analyses with a 4-year lag (e.g., 1990 dental history in relation to risk of POAG in 1994 – 1996 rather than 1994 dental history), as it is possible that there are delays in POAG diagnosis due to its insidious nature. Furthermore, to test whether dental issues may be just a marker of poor health status that may be related to POAG (e.g., diabetes), we conducted analyses on a subset of participants after excluding those with diabetes, those who were obese, those who smoked ≥ 30 pack-years, those who had reported no physician exams and those who reported having had a physician exam for medical concerns. As dentist participants may best report their dental history, we also conducted sensitivity analyses restricted to dentist participants to evaluate the robustness of findings. Finally, as dental history differed by race, we conducted an additional analysis restricted to Caucasians to evaluate whether any associations with dental history may be due to racial / socioeconomic differences that we could not measure.

5.5 Results

During 528,089 person-years of follow-up accrued over 26 years, we identified 485 incident POAG cases. Those with fewer teeth or who reported lost teeth in the most recent questionnaire (i.e., in the recent past 2 years) were older, had greater history of periodontal disease and greater number of teeth treated with root canals (Table 5.1). They were also more likely to be of African or Asian ancestry, to have a family history of glaucoma, to have a history of diabetes and heavy smoking and to consume more caffeine. They also exercised less and had higher BMI. These differences were adjusted for in multivariable analyses.

Compared to age-adjusted analyses, the multivariable analyses for number of teeth and POAG showed similar associations. We included 408 cases, after excluding those with missing number of teeth. Overall, we observed no linear associations between the number of teeth and all POAG or for other POAG subtypes (p for trend≥0.11 across outcomes; Table 5.2).

Table 5.1: Age and age-adjusted updated characteristics of total person-time of follow-up (528,089 person-years of follow-up), accrued from 1986 to 2012 among eligible participants 40 years and older*

	Number of teeth			Teeth lost in recent 2 years	
	17+	1-16	0	0	1+
Person-time, %	92.5	6.4	1.1	90.0	10.0
Age	61.4 ± 10.6	69.7 ± 9.3	69.1 ± 8.9	62.7 ± 10.3	67.8 ± 9.9
African-American, %	0.5	1.0	0.3	0.5	0.4
Asian-American, %	1.4	1.9	0.4	1.3	1.7
Family history of glaucoma, %	11.7	12.1	14.9	11.8	11.9
Cataract diagnosis or extraction, %	13.9	15.2	12.8	15.3	16.1
Age-related macular degeneration diagnosis, %	3.1	3.5	2.9	3.5	3.9
Diabetes, %	5.7	8.4	9.7	6.1	8.2
Hypertension, %	34.4	37.1	35.1	36.2	38.4
Number of eye exams reported†	7.0 ± 3.1	6.2 ± 3.1	5.1 ± 3.1	6.5 ± 3.2	6.3 ± 3.2
Alcohol intake (grams per day)	11.1 ± 13.7	11.1 ± 14.9	10.5 ± 13.9	11.1 ± 13.5	11.1 ± 13.9
Caffeine intake (milligrams per day)	224.6 ± 212.9	276.8 ± 236.1	293.4 ± 262.4	222.6 ± 205.7	254.1 ± 223.6
Body mass index (kg/m^2)	25.5 ± 3.1	26.1 ± 3.3	25.9 ± 3.3	25.5 ± 3.1	26.1 ± 3.3
≥30 years of pack-years of smoking, %	15.3	34.3	36.2	15.4	25.1
Highest quartile of physical activity, %	28.3	23.2	24.3	28.9	25.3
Updated number of natural teeth	24.0 ± 2.3	10.6 ± 5.0	0.0 ± 0.0	23.5 ± 3.8	20.4 ± 5.0
Periodontal disease diagnosed in past 2 years, %	9.6	23.8	16.0	8.3	22.1
Number of teeth lost in past 2 years‡	0.1 ± 0.4	1.0 ± 2.2	0.2 ± 1.4	0.0 ± 0.0	1.6 ± 1.6
Cumulative number of teeth with root canals$	1.6 ± 2.0	3.2 ± 2.4	1.4 ± 1.9	1.6 ± 2.0	2.7 ± 2.4

* Values are means ± SD or percentages for the entire total accumulated person-time of follow-up and are standardized to the age distribution of the total person-time, unless otherwise noted. Characteristics of person-time were updated every two years and accumulated over follow-up.

† As of the last follow-up period: number reported out of a maximum of 11 total exams over follow-up

‡ Among person-time accrued from 1988 (when number of teeth lost was first asked) to 2012

$ Among person-time accrued from 1996 (when number of teeth with root canal treatment was first asked) to 2012

Table 5.2: Multivariable-adjusted* relative risks (95% confidence intervals) for updated number of natural teeth in relation to risk of primary open-angle glaucoma (1986 – 2012)

	Updated number of natural teeth					
	25+	**17-24**	**11-16**	**1-10**	**0**	**P trend**
Primary analyses						
All cases (n=408 cases)	243	118	24	14	9	
Person-years	309,405	98,302	18,733	9,588	4,807	
ALL: Age-adjusted	1.00 (ref)	1.19 (0.95, 1.50)	1.01 (0.65, 1.57)	1.21 (0.68, 2.17)	1.47 (0.73, 2.95)	0.17
ALL: Multivariable-adjusted*	1.00 (ref)	1.21 (0.96, 1.53)	1.00 (0.64, 1.56)	1.19 (0.66, 2.14)	1.28 (0.63, 2.61)	0.26
Secondary analyses by IOP at diagnosis						
Cases of HTG† (n=292 cases)	177	79	18	11	7	
HTG†: Age-adjusted	1.00 (ref)	1.08 (0.82, 1.42)	0.98 (0.59, 1.64)	1.19 (0.61, 2.32)	1.35 (0.61, 2.96)	0.44
HTG†: Multivariable-adjusted *	1.00 (ref)	1.10 (0.83, 1.46)	0.98 (0.58, 1.65)	1.20 (0.61, 2.36)	1.19 (0.53, 2.68)	0.51
Cases of NTG† (n=116 cases)	66	39	6	3	2	
NTG†: Age-adjusted	1.00 (ref)	1.52 (1.00, 2.30)	1.09 (0.46, 2.61)	1.26 (0.38, 4.14)	1.99 (0.44, 9.02)	0.16
NTG†: Multivariable-adjusted *	1.00 (ref)	1.59 (1.03, 2.45)	1.04 (0.42, 2.56)	1.35 (0.40, 4.57)	2.01 (0.41, 9.86)	0.14
Secondary analyses by type of visual field loss						
Cases of Peri-POAG‡ (n=221 cases)	126	65	18	8	4	
Peri-POAG‡: Age-adjusted	1.00 (ref)	1.26 (0.92, 1.73)	1.38 (0.81, 2.35)	1.17 (0.52, 2.62)	1.10 (0.39, 3.11)	0.22
Peri-POAG‡: Multivariable-adjusted *	1.00 (ref)	1.22 (0.89, 1.68)	1.25 (0.73, 2.16)	1.12 (0.50, 2.53)	0.95 (0.33, 2.73)	0.43
Cases of Para-POAG‡ (n=120 cases)	70	39	4	3	4	
Para-POAG‡: Age-adjusted	1.00 (ref)	1.46 (0.96, 2.22)	0.72 (0.26, 2.00)	1.07 (0.33, 3.47)	2.86 (1.00, 8.20)	0.16
Para-POAG‡: Multivariable-adjusted *	1.00 (ref)	1.56 (1.01, 2.41)	0.74 (0.26, 2.11)	1.21 (0.36, 4.04)	2.88 (0.96, 8.60)	0.11

* All multivariable analyses were stratified by age in months and period at risk, and they were adjusted for the following variables: ancestry (African-American, Asian-American, all others), family history of glaucoma, self-reported history of cataract diagnosis or extraction, age-related macular degeneration, hypertension, diabetes, body mass index (22-23, 24-25, 26-27, 28-29, 30+ kg/m^2), cumulatively averaged intakes of alcohol (g/day) and caffeine (mg/day), dietary nitrate intake (mg/day), pack-years of smoking (1-9, 10-19, 20-29, 30+ pack-years), physical activity (quartiles of MET-hours [metabolic equivalents] / week), recent report of physician exam (for health maintenance / for medical concerns / no report of physical exam), updated number of eye exams reported during follow-up

†HTG=High tension primary-open angle glaucoma, based on the maximum untreated intraocular pressure (IOP) at diagnosis (IOP > 21 mm Hg); NTG=Normal tension glaucoma (IOP ≤ 21 mm Hg)

‡ Peri-POAG=Primary open-angle glaucoma with peripheral visual field (VF) loss, based on VF loss pattern as of the earliest reliable VF at diagnosis that was reproduced at the latest reliable VF. Cases with advanced VF loss at diagnosis (n=67) who could not be categorized based on initial presenting VF loss as either peripheral VF loss only or early paracentral VF loss were censored during analyses. See Methods for how cases were categorized according to initial presenting VF loss.

Table 5.3: Multivariable-adjusted* relative risks (95% confidence intervals) for incident periodontal disease in relation to risk of primary open-angle glaucoma (1986 – 2012)

	Periodontal disease status		
	Never diagnosed	**Diagnosed in distant past (>2 years)**	**Diagnosed in past 2 years**
Primary analyses			
All cases (n=485 cases)	259	158	68
Person-years	298,154	174,720	55,215
ALL: Age-adjusted	1.00 (ref)	0.80 (0.64, 0.99)	1.13 (0.85, 1.51)
ALL: Multivariable-adjusted*	1.00 (ref)	0.79 (0.63, 0.98)	1.15 (0.86, 1.55)
Secondary analyses by IOP at diagnosis			
Cases of HTG† (n=341 cases)	189	107	45
HTG†: Age-adjusted *	1.00 (ref)	0.83 (0.64, 1.07)	1.04 (0.74, 1.48)
HTG†: Multivariable-adjusted *	1.00 (ref)	0.82 (0.63, 1.07)	1.05 (0.73, 1.49)
Cases of NTG† (n=144 cases)	70	51	23
NTG†: Age-adjusted *	1.00 (ref)	0.75 (0.51, 1.11)	1.36 (0.81, 2.29)
NTG†: Multivariable-adjusted *	1.00 (ref)	0.70 (0.46, 1.06)	1.45 (0.85, 2.49)
Secondary analyses by type of visual field loss			
Cases of Peri-POAG‡ (n=260 cases)	139	86	35
Peri-POAG‡: Age-adjusted *	1.00 (ref)	0.89 (0.66, 1.19)	0.97 (0.65, 1.44)
Peri-POAG‡: Multivariable-adjusted *	1.00 (ref)	0.82 (0.61, 1.11)	0.90 (0.60, 1.36)
Cases of Para-POAG‡ (n=147 cases)	82	42	23
Para-POAG‡: Age-adjusted *	1.00 (ref)	0.63 (0.42, 0.94)	1.51 (0.91, 2.49)
Para-POAG‡: Multivariable-adjusted *	1.00 (ref)	0.66 (0.43, 1.00)	1.61 (0.95, 2.72)

* All multivariable analyses were stratified by age in months and period at risk, and they were adjusted for the following variables: ancestry (African-American, Asian-American, all others), family history of glaucoma, self-reported history of cataract diagnosis or extraction, age-related macular degeneration, hypertension, diabetes, body mass index (22-23, 24-25, 26-27, 28-29, 30+ kg/m^2), cumulatively averaged intakes of alcohol (g/day) and caffeine (mg/day), dietary nitrate intake (mg/day), pack-years of smoking (1-9, 10-19, 20-29, 30+ pack-years), physical activity (quartiles of MET-hours [metabolic equivalents] / week), recent report of physician exam (for health maintenance / for medical concerns / no report of physical exam), updated number of eye exams reported during follow-up

†HTG=High tension primary-open angle glaucoma, based on the maximum untreated intraocular pressure (IOP) at diagnosis (IOP > 21 mm Hg); NTG=Normal tension glaucoma (IOP ≤ 21 mm Hg)

‡ Peri-POAG=Primary open-angle glaucoma with peripheral visual field (VF) loss; Para-POAG=Primary open-angle glaucoma with paracentral VF loss. This classification is based on VF loss pattern as of the earliest reliable VF at diagnosis that was reproduced at the latest reliable VF. Cases with advanced VF loss at diagnosis (n=78) who could not be categorized based on initial presenting VF loss as either peripheral VF loss only or early paracentral VF loss were censored during analyses. See Methods for how cases were categorized according to initial presenting VF loss.

Compared with no report of periodontal disease during follow-up, a report of a diagnosis of periodontal disease in the past 2 years was not associated with increased POAG risk. Interestingly, a report a diagnosis of periodontal disease during follow-up but not in the past 2 years was inversely associated with overall POAG: 0.79 (95% CI, 0.63, 0.98; p=0.03) (Table 5.3). Recent or past history of periodontal disease was not significantly associated with any of the other subtypes of POAG.

We conducted analyses from 1988 among those with at least 1 or more teeth (n=361 POAG cases) to evaluate tooth loss and POAG risk. Compared with those not reporting any teeth lost during follow-up, the MVRR was 1.45 (95% CI, 1.06, 1.97; p=0.02) for reporting 1+ teeth lost within the past 2 years and 1.07 (95% CI, 0.78, 1.46; p=0.69) for reporting 1+ teeth lost sometime during follow-up but not within the past 2 years (Table 5.4). Furthermore, with a report in the past 2 years of both 1+ teeth being lost and periodontal disease with bone loss, the adverse association was stronger (MVRR =1.85; 95% CI, 1.07, 3.18; p=0.03) than with one or more teeth being lost without periodontal disease (MVRR =1.33; 95% CI, 0.94, 1.89; p=0.11). Tooth loss in the past 2 years was also significantly associated with NTG (MVRR= 1.93; 95% CI, 1.09, 3.43; p=0.02) and Para-POAG (MVRR= 2.27; 95% CI, 1.32, 3.88; p=0.003) (Table 5.4). However, the p for heterogeneity between HTG and NTG (p=0.46) or between Peri-POAG and Para-POAG (p=0.36) were not significant.

In an analysis from 1996 (n= 277 POAG cases), the number of teeth with root canal treatment was not associated with any of the outcomes (Table 5.5). The p for trend for increasing number of teeth with such treatment was 0.82 for all POAG, and it was ≥ 0.16 for all other subtypes

Table 5.4: Multivariable-adjusted* relative risks (95% confidence intervals) for number of incident teeth lost in relation to risk of primary open-angle glaucoma (1988 – 2012)

	Number of teeth lost				
	0	**1+ lost in distant past (>2 years)**	**1+ lost in past 2 years**	**1+ lost in past 2 years with no recent periodontal disease**	**1+ lost in past 2 years with recent periodontal disease**
Primary analyses					
All cases (n=364 cases)	251	57	56	40	16
Person-years	281,777	47,255	34,863	26,827	7,981
ALL: Age-adjusted	1.00 (ref)	1.08 (0.79, 1.47)	1.43 (1.06, 1.94)	1.34 (0.95, 1.90)	1.73 (1.01, 2.95)
ALL: Multivariable-adjusted*	1.00 (ref)	1.07 (0.78, 1.46)	1.45 (1.06, 1.97)	1.33 (0.94, 1.89)	1.85 (1.07, 3.18)
Secondary analyses by IOP at diagnosis					
Cases of HTG† (n=260 cases)	187	34	39	27	12
HTG†: Age-adjusted *	1.00 (ref)	0.88 (0.59, 1.30)	1.34 (0.93, 1.91)	1.23 (0.81, 1.87)	1.67 (0.91, 3.09)
HTG†: Multivariable-adjusted *	1.00 (ref)	0.85 (0.57, 1.27)	1.32 (0.91, 1.90)	1.19 (0.78, 1.82)	1.74 (0.93, 3.25)
Cases of NTG† (n=104 cases)	64	23	17	13	4
NTG†: Age-adjusted *	1.00 (ref)	1.63 (0.97, 2.71)	1.71 (0.97, 3.01)	1.65 (0.88, 3.10)	1.94 (0.66, 5.69)
NTG†: Multivariable-adjusted *	1.00 (ref)	1.65 (0.97, 2.81)	1.93 (1.09, 3.43)	1.81 (0.95, 3.44)	2.46 (0.82, 7.39)
Secondary analyses by type of visual field loss					
Cases of Peri-POAG‡ (n=197 cases)	134	35	28	19	9
Peri-POAG‡: Age-adjusted *	1.00 (ref)	1.24 (0.83, 1.86)	1.29 (0.84, 1.98)	1.16 (0.70, 1.92)	1.71 (0.83, 3.53)
Peri-POAG‡: Multivariable-adjusted *	1.00 (ref)	1.17 (0.78, 1.76)	1.21 (0.79, 1.87)	1.08 (0.65, 1.79)	1.67 (0.80, 3.48)
Cases of Para-POAG‡ (n=107 cases)	71	16	20	15	5
Para-POAG‡: Age-adjusted *	1.00 (ref)	1.13 (0.62, 2.03)	2.04 (1.21, 3.41)	1.88 (1.05, 3.35)	2.71 (1.06, 6.94)
Para-POAG‡: Multivariable-adjusted *	1.00 (ref)	1.23 (0.66, 2.27)	2.27 (1.32, 3.88)	2.02 (1.11, 3.68)	3.52 (1.31, 9.43)

* All multivariable analyses were stratified by age in months and period at risk, and they were adjusted for the following variables: ancestry (African-American, Asian-American, all others), family history of glaucoma, self-reported history of cataract diagnosis or extraction, age-related macular degeneration, hypertension, diabetes, body mass index (22-23, 24-25, 26-27, 28-29, 30+ kg/m^2), cumulatively averaged intakes of alcohol (g/day) and caffeine (mg/day), dietary nitrate intake (mg/day), pack-years of smoking (1-9, 10-19, 20-29, 30+ pack-years), physical activity (quartiles of MET-hours [metabolic equivalents] / week), recent report of physician exam (for health maintenance / for medical concerns / no report of physical exam), updated number of eye exams reported during follow-up

†HTG=High tension primary-open angle glaucoma, based on the maximum untreated intraocular pressure (IOP) at diagnosis (IOP > 21 mm Hg); NTG=Normal tension glaucoma (IOP ≤ 21 mm Hg)

‡ Peri-POAG=Primary open-angle glaucoma with peripheral visual field (VF) loss; Para-POAG=Primary open-angle glaucoma with paracentral VF loss. This classification is based on VF loss pattern as of the earliest reliable VF at diagnosis that was reproduced at the latest reliable VF. Cases with advanced VF loss at diagnosis (n=60) who could not be categorized based on initial presenting VF loss as either peripheral VF loss only or early paracentral VF loss were censored during analyses. See Methods for how cases were categorized according to initial presenting VF loss

Table 5.5: Multivariable-adjusted* relative risks (95% confidence intervals) for number of teeth with root canal treatment in relation to risk of primary open-angle glaucoma (1996 – 2012)

	Updated number of total teeth with root canals				
	0	**1**	**2-4**	**5+**	**P trend**
Primary analyses					
All cases (n=277 cases)	99	64	90	24	
Person-years	102,837	58,478	80,290	20,515	
ALL: Age-adjusted	1.00 (ref)	1.01 (0.73, 1.39)	1.03 (0.77, 1.38)	1.04 (0.65, 1.64)	0.94
ALL: Multivariable-adjusted*	1.00 (ref)	1.02 (0.73, 1.41)	1.03 (0.77, 1.39)	1.08 (0.68, 1.72)	0.82
Secondary analyses by IOP at diagnosis					
Cases of HTG† (n=170 cases)	58	45	52	15	
HTG†: Age-adjusted *	1.00 (ref)	1.23 (0.83, 1.84)	1.01 (0.69,1.49)	1.06 (0.59, 1.90)	0.98
HTG†: Multivariable-adjusted *	1.00 (ref)	1.27 (0.85, 1.89)	1.00 (0.68,1.48)	1.14 (0.63, 2.06)	0.85
Cases of NTG† (n=107 cases)	41	19	38	9	
NTG†: Age-adjusted *	1.00 (ref)	0.67 (0.37, 1.20)	1.05 (0.66, 1.66)	0.99 (0.47, 2.09)	0.89
NTG†: Multivariable-adjusted *	1.00 (ref)	0.67 (0.37, 1.22)	1.09 (0.68, 1.73)	0.99 (0.46, 2.12)	0.90
Secondary analyses by type of visual field loss					
Cases of Peri-POAG‡ (n=152 cases)	60	36	46	10	
Peri-POAG‡: Age-adjusted *	1.00 (ref)	0.94 (0.61,1.43)	0.84 (0.57, 1.25)	0.71 (0.36, 1.42)	0.21
Peri-POAG‡: Multivariable-adjusted *	1.00 (ref)	0.90 (0.59,1.39)	0.82 (0.55, 1.23)	0.69 (0.34, 1.40)	0.19
Cases of Para-POAG‡ (n=79 cases)	25	16	30	8	
Para-POAG‡: Age-adjusted *	1.00 (ref)	0.92 (0.47, 1.79)	1.41 (0.81, 2.43)	1.43 (0.63, 3.24)	0.20
Para-POAG‡: Multivariable-adjusted *	1.00 (ref)	0.91 (0.46, 1.80)	1.54 (0.88, 2.69)	1.47 (0.64, 3.40)	0.16

* All multivariable analyses were stratified by age in months and period at risk, and they were adjusted for the following variables: ancestry (African-American, Asian-American, all others), family history of glaucoma, self-reported history of cataract diagnosis or extraction, age-related macular degeneration, hypertension, diabetes, body mass index (22-23, 24-25, 26-27, 28-29, 30+ kg/m^2), cumulatively averaged intakes of alcohol (g/day) and caffeine (mg/day), dietary nitrate intake (mg/day), pack-years of smoking (1-9, 10-19, 20-29, 30+ pack-years), physical activity (quartiles of MET-hours [metabolic equivalents] / week), recent report of physician exam (for health maintenance / for medical concerns / no report of physical exam), updated number of eye exams reported during follow-up

†HTG=High tension primary-open angle glaucoma, based on the maximum untreated intraocular pressure (IOP) at diagnosis (IOP > 21 mm Hg); NTG=Normal tension glaucoma (IOP ≤ 21 mm Hg)

‡ Peri-POAG=Primary open-angle glaucoma with peripheral visual field (VF) loss; Para-POAG=Primary open-angle glaucoma with paracentral VF loss. This classification is based on VF loss pattern as of the earliest reliable VF at diagnosis that was reproduced at the latest reliable VF. Cases with advanced VF loss at diagnosis who could not be categorized based on initial presenting VF loss as either peripheral VF loss only or early paracentral VF loss were censored during analyses. See Methods for how cases were categorized according to initial presenting VF loss.

In sensitivity analyses of tooth loss in the past 2 years and incident POAG, stronger associations were observed when in multivariable analyses, we further adjusted for current number of teeth and periodontal bone loss status: MVRR= 1.54 (95% CI, 1.11, 2.13; p=0.01) (not shown in tables). When we evaluated age subgroups, we observed that associations tended to be stronger in those <65 years (117 POAG cases; MVRR= 2.13, 95% CI, 1.21, 3.76; p=0.01) versus those who were 65 years and older (247 POAG cases; MVRR=1.25, 95% CI, 0.87, 1.80; p=0.23), with a borderline significant interaction (p for interaction by age = 0.06). Associations for tooth loss and incident POAG were attenuated when we introduced a 4-year lag period (309 POAG cases; MVRR=1.16, 95% CI, 0.80, 1.68; p=0.44). However, associations were only slightly attenuated when we restricted analyses to Caucasians (349 POAG cases; MVRR= 1.40, 95% CI, 1.02, 1.93; p=0.04) or to dentist participants (210 POAG cases; MVRR=1.45, 0.93, 2.24; p=0.10), and associations seemed stronger in those who were relatively healthy, defined as those who all reported physical exams for health maintenance only (versus for medical concerns), who did not report any diabetes mellitus or obesity, and who reported less than a 30 pack-year history of smoking (157 POAG cases; MVRR= 2.13, 95% CI, 1.33, 3.39; p=0.002).

5.6 Discussion

Primary open-angle glaucoma is a neurodegenerative disease that can lead to blindness and for which there are few established risk factors. In this large long-term prospective study among male health professionals, we observed no associations with number of natural teeth, history of periodontitis or number of teeth with root canal treatment. However, we observed that loss of at least one tooth reported in the recent past 2 years was associated with a modestly increased risk of POAG, in particular, tooth loss accompanied by periodontal disease with bone loss in the recent past 2 years showed the strongest associations, although the confidence intervals for both estimates of associations were broad. Given that in adults 40+ years old, the most common cause of tooth loss is periodontal disease,[1075, 1076] this suggests that oral infections that lead to periodontal disease with bone loss severe enough to lead to tooth loss, may be associated with transient increases in risk of POAG. Because this was the first study to link recent tooth loss with POAG, and some of the significant results may be due to chance, these findings should be interpreted with caution and confirmed with other studies.

To date, there has been scarce data linking glaucoma to the oral microbiome.[663, 1077, 1078] One clinic-based case-control study of 103 African-American subjects[663] observed that those with oral bacteria loads in the upper quartile were over three times more likely to have glaucoma and that glaucoma cases had significantly fewer teeth, especially in older persons.[1064] In addition, they observed that in two glaucoma animal models[663] administration of low dose subcutaneous lipopolysaccharide to simulate the condition of chronic subclinical bacterial infection, exacerbated glaucomatous neurodegeneration. The possible mechanisms may be related to upregulation of complement system and toll-like receptor 4 signaling activity along with microglial activation in the optic nerve,[663] which occur early in the glaucomatous process.[1079]

In addition to a possible immune-related response in the optic nerve from oral infections, other mechanisms may be operative, especially IOP-independent mechanisms, as we did not observe differences in associations for high tension versus normal tension glaucoma. Another IOP-independent mechanism that may explain the link between oral health and glaucoma may be systemic endothelial cell dysfunction. Periodontitis, the most common oral infection, induces a subclinical systemic inflammatory response leading to endothelial cell dysfunction, and such dysfunction can be reversed over several months with

periodontal disease treatment.[1060-1062] Endothelial dysfunction can lead to impaired flow-mediated vasodilation that affects blood flow to the optic nerve, which has been associated with POAG across the spectrum of IOP.[1080, 1081] Our observation of somewhat stronger associations between recent tooth loss and POAG with early paracentral loss, a form of glaucoma linked to vascular endothelial dysfunction, [1082, 1083] further supports this mechanism. The attenuated association with past tooth loss that occurred >2 years versus that reported in the past 2 years may reflect the possibility that occurrences of tooth loss or periodontitis that occurred > 2 years in the past would likely have been resolved or treated and that such treatment may have led to improvement in endothelial function and long-term better maintenance of good oral health,[1061, 1062] unlike a recent bout of tooth loss that is accompanied by periodontitis. However, because this result may be due to chance, and our interpretation may be speculative, the modest associations observed need confirmation in studies with greater number of exposed cases.

We observed no associations between number of teeth with root canal treatment and POAG. Root canal treatment generally reflects prior endodontic inflammation, stemming from dental caries, and occasionally, root canal therapies are used to salvage teeth due to a variety of other reasons. The pathophysiology and microbes related to endodontic inflammation are different from periodontal disease; in particular, the dysbiosis associated with periodontitis evokes a strong and direct immune response, whereas the dysbiosis associated with caries promotes demineralization through acidogenic and aciduric mechanisms.[1084] Furthermore, endodontic inflammation is less common than periodontal disease, and there is much less evidence for the systemic impact of endodontic inflammation.[1085, 1086]

Our study had a few limitations. Because we were not able to conduct repeated eye exams on our participants over a 26-year period, we relied on participants' self-report of glaucoma confirmed with medical records. While such a case-ascertainment method would lead to under ascertainment of glaucoma, methodologically, bias in the estimation of a relative risk is minimal if the outcome is highly specific (such as our definition of POAG that required reproducible VF loss on reliable VFs), and the ascertainment of disease is unlikely to be related to oral health.[1087] To help ensure that ascertainment of glaucoma itself would not be different by oral health status, we included only those who reported eye exams in analyses, adjusted for the following factors: the number of eye exams reported during follow-up; other

eye diseases, and whether participants had physician exams for either symptoms or health maintenance. We also censored participants who did not respond to oral health questions on two consecutive questionnaires. Furthermore, to evaluate the possibility of reverse causality, we conducted analyses of whether having POAG itself may later lead to greater tooth loss. We identified 8310 events of incident tooth loss from 1988 to 2012; the multivariable RR for incident tooth loss for prevalent POAG versus no POAG was 0.84 (95% CI= 0.48, 1.46), indicating little support for reverse causation or coexistence of frequent eye exams and frequent dental exams explaining the association. Oral health measures were self-reported in our study; however, the self-reports were validated to be accurate when compared against dental radiographic findings in a subset of our participants,[1070-1072] and similar, although non-significant, associations with recent teeth lost were observed among dentists in our cohort. Given that our participants were all males and predominantly Caucasian, the magnitude of associations observed may not be generalizable to the general population. In our restricted analyses that included only Caucasians, the association with recent tooth loss was slightly attenuated, indicating there might be some differences by race. However, our results are consistent with the findings of Astafurov *et al*.[663] and Polla *et al*.[1064] reported among African-Americans, implicating a role for oral health in POAG. More studies in women and other racial/ethnic groups may help to further shed light on this link, as prevalence of periodontal disease and dental problems differ by gender and race.[1088, 1089]

Our study has a number of strengths. The prospective design allowed us to examine the relation between oral health and incident POAG and allowed us to minimize recall bias or bias that may arise with including prevalent glaucoma cases if glaucoma treatment could modify the association between oral health and POAG. The number of teeth and periodontal disease status was assessed every 2 years over 25+ years. The results point to periodontal disease, as opposed to tooth loss related to dental caries or other causes, as the key dental exposure linked to POAG (Table 4). The association observed with number of teeth lost is unlikely to be due to tooth loss being a mere marker of overall poor health that may also be linked to glaucoma. After excluding those with diabetes, those who were obese, those who smoked ≥ 30 pack-years, those who had reported no physician exams or reported having had a physician exam due to medical concerns versus only for health maintenance, the association between number of teeth lost and POAG was robust, further supporting an etiologic link between dental pathology and POAG.

In conclusion, while the number of natural teeth and any periodontal disease was not associated with risk of POAG, we observed an adverse association between recent tooth loss, combined with recent periodontal disease, and risk of POAG. The results of this study raise important questions that could be addressed in future studies: how dental pathology, particularly severe periodontitis, may affect glaucoma pathology and whether prompt attention to periodontal disease might alter the development of glaucoma. Because this is the first study to link recent tooth loss with POAG, these findings should be interpreted with caution and confirmed with other studies.

Section 3 – Animal Research

Chapter 6 – The Microbiome is Protective in Optic Nerve Crush in Mice

6.1 Chapter Overview and Introduction

Section 2 has addressed the epidemiological link between microbiome related illnesses and glaucoma finding that IBS may be a risk factor for glaucoma, and that there may also be an association between oral health outcomes and glaucoma. This following chapter presents the findings linking microbiome states to outcomes in an ONC model of glaucoma.

Glaucoma is neurodegenerative disorder that leads to the death of Retinal Ganglion Cells (RGCs). It remains one of the largest causes of irreversible blindness world wide[1037], and to date only one clinically significant modifiable risk factor has demonstrated any role for therapeutic management. In addition to the research presented in this thesis, there is research to suggest that the microbiome may play a role in glaucoma[245, 663]. Another group has shown that oral health may impact on glaucoma prevalence[663]. Furthermore, recent research has demonstrated a T cell mediated effect, whereby microbiome priming of the immune system was shown to contribute to the neurodegenerative effects seen in an ocular hypertension model[245].

Understanding of host-microbiome interactions has exploded in the past two decades. Since the work of Sudo *et al* in 2004[513], particular interest has been paid to the microbiome's effects on the central nervous system. It is well known that the microbiome is formative for the immune system[412, 413], and from this alone it is clear that the microbiome is involved in the development of many body systems. In the CNS, Synaptic protein expression[515], blood brain barrier integrity[555] and neurogenesis[977] all appear to be regulated by the microbiome. Similarly, and probably not surprisingly, the neuro-immune system is modulated by the microbiome[540-542]. One of the earliest findings regarding microbiome-CNS effects though, implicates so many of the CNS's processes, namely that BDNF expression is reduced in the absence or disturbance of the microbiome[513], a finding that has since been replicated by a number of groups[515, 516, 518].

BDNF is a potent neuroprotective peptide that has been shown to consistently protect RGCs in animal models of glaucoma[340-347]. Similarly, there have been reports that elevating central levels of BDNF can augment the effects of BDNF at the retina[355, 356]. If administering exogenous BDNF to the retina has neuroprotective effects, it stands to reason that biological

processes that elevate the endogenous levels of BDNF should also have effects on the survival of RGC's in an optic nerve crush model (ONC) of glaucoma.

It was hypothesized that GF mice would have worse RGC survival after ONC compared to mice with normal microbiome. Further we hypothesized that this protective mechanism may be modulated through BDNF mediated mechanisms. Finally, it was hypothesized that Intraocular injections of BDNF and colonization with a probiotic strain of lactobacillus would have neuroprotective effects in this ONC model.

6.2 Methods

Overview

Germ Free (GF), Specific Pathogen Free (SPF), and Conventionalized Germ Free (CON) mice were subjected to an ONC and allowed to survive until their retinae were harvested for analysis (up to 3 days for protein analysis, 1 week for qPCR and 5 weeks for cell survival analysis). Immunohistochemistry was used to examine the cell survival, and qPCR and ELISA protein analysis were used to quantify the BDNF levels in the retina, at various time points after the ONC. A further cohort of GF mice were treated with live or heat-killed *Lactobacillus* probiotic, and its effects on cell survival after ONC were quantified. Finally, a cohort of GF and SPF mice were also received an injection of BDNF protein at the time of ONC and its effects were compared to mice who received a placebo injection.

Mice

GF and SPF C57BL/6J female and male mice (8 to 10 weeks old) were used. GF mice were taken from 3rd or greater generation germ-free mice (i.e. these mice were not exposed to microbes even indirectly through mothers). GF mice were raised in transparent plastic isolators. All animals were housed in the animal facilities at the Singapore Experimental Medical Centre.

A cohort of GF mice were then conventionalized with the microbiome from SPF mice. 8- to 10-week-old C57BL/6J GF mice received faecal matter from the pathogen-free mice through a single gavage and then were allowed to breed. The CON mice from the subsequent generations were used for experiments.

All animals were maintained on autoclaved chow diet, given sterile drinking water ad libitum, and kept under 12-hour light/dark cycles.

The protocols were approved by the SingHealth Institutional Animal Care Use Committee (IACUC approval number: 2015/SHS/1022) in accordance with the National Advisory Committee For Laboratory Animal Research guidelines, as specified under Singapore legislation in the Animals and Birds Act Chapter 7, Section 80 – Animals and Birds (Care and use of animals for scientific purposed) rules, 2004[1090]. All animal handling and experiments were also undertaken in accordance with the Association for Research in Vision and Ophthalmology statement for use of Animals in Ophthalmic and Vision Research[1091].

Probiotic Treatment

Lactobacillus plantarum PS128 powder was dissolved in warmed normal saline to a concentration of 5x10^9 CFU/mL. GF C57BL/6J mice (6 to 8 weeks old) were gavaged with 200 µL of the probiotic solution every day for 2 weeks prior to experimentation, and then every day until the end of experimentation. On the day of experiments, mice were gavaged at least 2 hours prior to experiments.

Heat-Killed *L. plantarum* PS128 was created by preparing the probiotic in saline as described above, and then exposed to a hot water bath at 80 degrees Celsius for 8 minutes. Probiotic's were then confirmed to be heat killed by plating them on Aerobic and Anaerobic culture plates as is used typically within the facility to detect microbiome contamination.

Optic Nerve Crush

8-10 week old mice were used for all ONC procedures. ONC followed a previously published protocol described by Templeton *et al*.[907]

Under sterile conditions, Mice were placed on a heating pad and anaesthetised by inhaled isoflurane. Under an operating microscope the eye was grasped by the conjunctiva and rotated infero-nasally. Vanna's scissors were used to dissect the conjunctiva and the soft tissue, rotation of the eye to the inferonasal position allowed for the optic nerve to be visualised between the rectus muscles. On visualisation, cross action forceps (Dumont #N7 Cross Action Forceps, Roboz, USA) were applied to the optic nerve for 10 seconds and then released. The eye was then allowed to rotate back into place, the conjunctiva was laid back over the eye in its initial positioning and a drop of topical anaesthetic (Alcaine, Alcon, Canada) was applied to the conjunctiva. ONC was performed bilaterally at the same timepoint.

Mice were then placed in an isolation cage until they had regained consciousness, if there were any signs of pain after anaesthetic had worn off a second drop of topical anaesthetic was applied, and the mouse was monitored for ongoing signs of pain. Following recovery, mice were returned to their home cages. Mice were then kept in BSL-2 hoods until harvesting to minimize contamination over follow-up.

BDNF Injection

A separate cohort of mice were given injections of BDNF or vehicle. Under sterile conditions, recombinant human BDNF (Peprotech, USA) was dissolved in 1% Bovine Serum Albumin in 0.9% saline solution (BSA) to a concentration of 1μg/μL. Mice were arranged into two cohorts by animal house technicians blinded to the experimental design. A coin flip decision was made to determine which batch would receive the BDNF injection and which would receive the vehicle, BSA, injection. At the time of ONC, after the crush pressure had been removed, the conjunctiva was grasped with forceps and approximately 0.5-1mm posterior to the limbus a 10microliter injection of BDNF or BSA was injected using a custom-made Ito Microsyringe with 33guage needle.

Tissue Harvesting

Mice were euthanised by inhalation of CO_2 and confirmed with cervical dislocation. Eyes were immediately enucleated post mortem and stored, in Phosphate Buffered Saline (PBS), on ice until harvested (within 30 minutes).

Retinae were harvested under operating microscope (Figure 6.1). The eye was grasped with forceps and a small incision into the sclera, near the limbus, was made with a 20-gauge needle. This incision was then used as the starting point for circumlimbal dissection of the anterior eye from the 'eye cup'. Once the anterior eye had been removed, the retina was visualised and using a fine paintbrush the eyecup was peeled away from the pigmented epithelium of the eye cup. The optic nerve was severed at the optic nerve head and the retina was then able to be used for immunohistochemistry, RNA or protein analysis.

Retinae were harvested on day 0, day 3, day 7 and day 35 for immunohistochemistry-based cell survival analysis; day 0, day 1, day 3 and day 7 for qPCR; and day 0 and day 3 for protein analysis. Timepoints for protein and mRNA analysis were chosen based on previously reported peaks in BDNF expression after ONC[316].

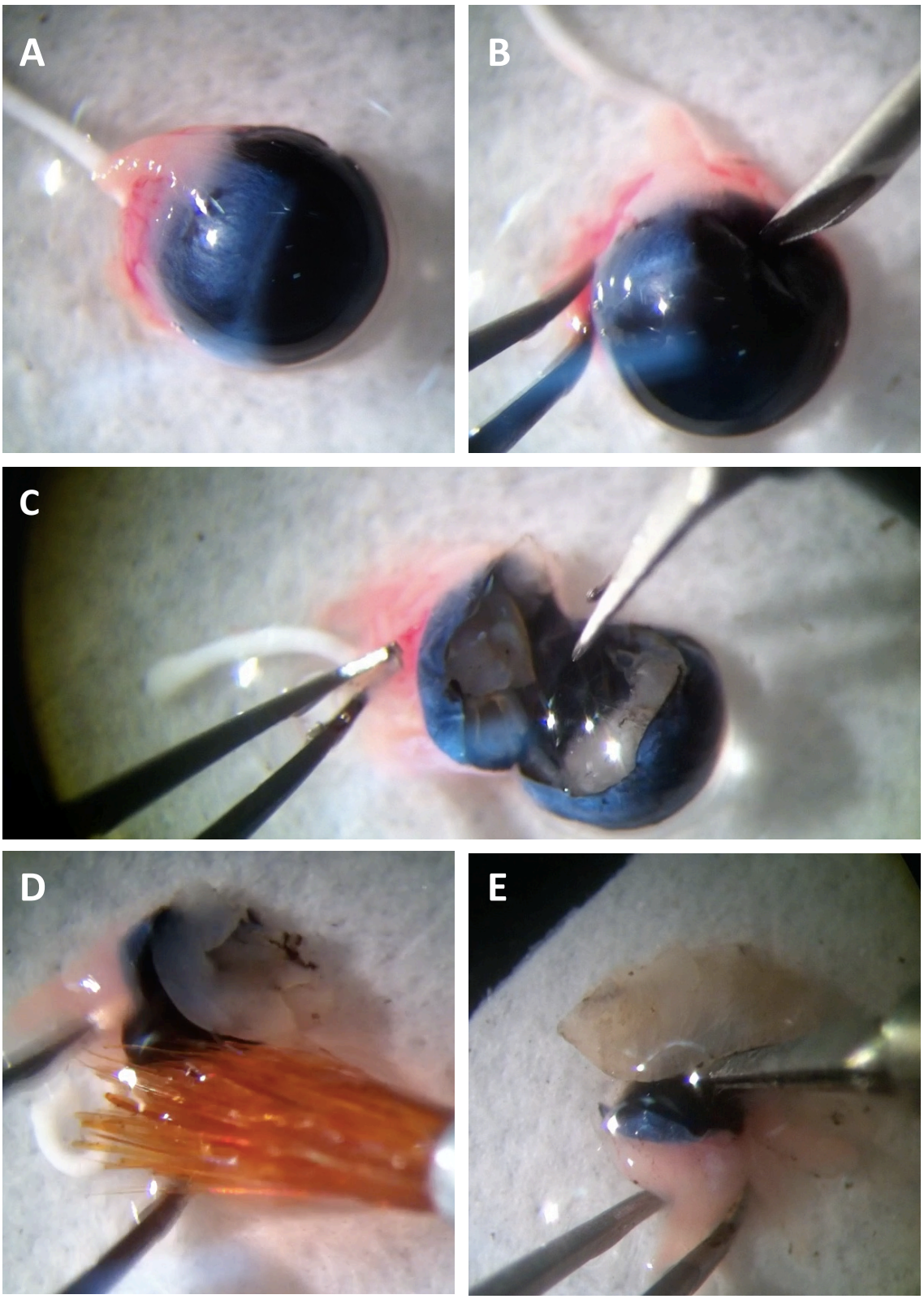


Figure 6.1: Representative images demonstrating retinal dissection from murine optic nerve cup, taken through operative microscope

The enucleated eye (A) is grasped with forceps, a small incision is made with a needle in the sclera near the limbus (B). This incision was then used as the starting point for circumlimbal dissection of the anterior eye from the 'eye cup' (C). The retina is peeled away from the eye cup with a fine paintbrush (D). The optic nerve is severed at the optic nerve head (E).

Immunohistochemistry

Fresh retinae were transferred to cell culture inserts (Millipore, NJ, USA), with ganglion cell layer facing up, for the purposes of wholemount staining. Retinae were not oriented and therefore the sampling was random without taking into account the characteristic RGC distribution within the mice retina. Retinae were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, MO, USA) for 1 hour. Retinae were washed with 1 X PBS three times each for 15 minutes with gentle shaking, at room temperature. The retinae were then blocked using blocking solution [5% BSA (Sigma-Aldrich, MO, USA) and 0.2% triton-x (Sigma-Aldrich, MO, USA) in 1X PBS] for 1 hour with gentle shaking, at room temperature. RGC density was estimated by rabbit anti-mouse RBPMS antibody, a marker specific for RGCs[1092, 1093] (1:500; GeneTex, CA, USA). The retinae were incubated with primary antibodies overnight at 4ºC and then thrice washed with 1 X PBS the following morning. Then the retinae were incubated with Fluorescent secondary antibody, Alexa-fluor A488 Donkey Anti rabbit IgG antibody (1:1000; life technologies, NY, USA) in 2%BSA and 0.2% triton in PBS overnight at 4ºC and washed with PBS as done above. To ensure correct orientation, retinae were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000; life technologies, NY, USA) for 1 hour and then finally washed in PBS before being mounted. Finally, the retinae were cut from the plastic casing of the Millipore filters and placed on glass slides, ganglion cell side facing up, a single drop of glycerol was used as mounting solution.

Imaging was performed at 40× magnification using confocal microscope (Leica TCS SP8; Olympus, NY, USA) at the Singapore Advanced Bioimaging Core (The Academia, Singapore). Ganglion cells were identified based on morphology and plane of focus of the wholemount. Images were taken by a masked observer at six random locations for each retina. Cell counts were performed by a masked observer using image analysis software (ImageJ, United States National Institutes of Health (NIH)).

mRNA Analysis (qPCR)

For the purposes of RNA analysis, fresh retina, after dissection, were transferred immediately to individual tubes containing ice cold TRIzol reagent (Invitrogen, USA). Samples were then lysed in TRIzol with a bead beating tissue lyser. These samples were then either

processed immediately into RNA or stored overnight at -20°C and processed into RNA the following day.

Following tissue lysis, chloroform was added to the sample in TRIzol solution and the samples were mixed and allowed to separate before being centrifuged at 12,000*g* for 10 mins at 4°C. Carefully the aqueous phase was removed and transferred to another Eppendorf tube. This phase was treated with 1:3 ratio of 70% ethanol and this solution was mixed by pipetting prior to transfer to RNA isolation columns. RNA isolation proceeded according to the manufacturer's instructions (RNeasy Mini kit; Qiagen, Netherlands). Following RNA isolation, residual genomic DNA was digested with DNase (DNase 1, amplification grade; life technologies, USA) to avoid false-positive bands after PCR. Then, the mRNA was converted into cDNA using the Superscript III first-strand synthesis system kit (life technologies, USA) using the manufacturer's instructions. The cDNA was then used for PCR. KAPA SYBR FAST qPCR master mix (life technologies, USA) was used as master mix for the PCR reaction.

The Primer3Plus online designing tool was used to design primers and these were then checked with the Primer-BLAST tool (NIH). For total BDNF the primers used were forward sequence, 5'- GGCCCAACGAAGAAAACCAT - 3', and reverse primer sequence, 5'-AGCATCACCCGGGAAGTGT -3'. For the housekeeping gene GAPDH the forward sequence, 5' –TTCCATCCTCCAGAAACCAG-3', was used with the reverse primer sequence, 5'-CCCTCGAACTAAGGGGAAAG-3'. The PCR reaction was performed using CFX96 Bio-Rad system (Bio-Rad, CA, USA). The cDNA template was denatured for ten minutes at 95°C before amplification was started. The reaction was stopped after 40 cycles. Each cycle consisted of 30 seconds at 95°C, 60 seconds at 60°C.

Protein Analysis

For the purposes of protein analysis, fresh retinae were snap frozen in Liquid Nitrogen (Two retina per sample). Samples were stored at -80°C for up to 2 weeks before being processed. Samples were lysed in RIPA protein lysis buffer with protease inhibitor [cOmplete, mini, EDTA free protease inhibitor cocktail (Sigma, USA)]. Protein concentrations of samples were quantified using the Bio-Rad Protein Assay Kit II (Bio-Rad, USA) according to the manufacturer's instructions. BDNF concentration of samples was determined with the BDNF E-max ELISA system (Promega, USA) according to the manufacturer's instructions.

Statistical Analysis

Timepoints for tissue collection were chosen based on previous studies which demonstrated that significant cell death occurs after ONC by 1 week and achieves stability by 1 month. Similarly, for protein and mRNA analysis it has been shown that day 3 appears to be the peak of BDNF elevation after ONC.

For each timepoint each condition was compared to each other within the investigation, and for each condition, each timepoint was compared to the other timepoints within the investigation (i.e. GF retina's would be compared to the SPF retinae from the same timepoint, and to other GF retinae at different timepoints, but not to the SPF retinae from different timepoints). Specifically for the BDNF injection study, there were 3 comparisons to be made: firstly the BDNF treated eyes were compared to the BSA treated eyes from the same kind of mouse, at the same timepoint; secondly, the BDNF treated eyes were compared between GF and SPF mice, and the BSA treated eyes were compared between the GF and SPF treated groups; finally, each condition was assessed longitudinally.

2-way ANOVA's were performed for each investigation. $p<0.05$ was defined as statistical significance. For cell survival studies, cell counts are normalized to the cell density of SPF mice at baseline, except for the probiotic study where graphs are displayed normalized to GF at baseline. Significant results within each timepoint are noted on each graph. Bars are displayed as means $\pm$ standard error of the mean (SEM).

6.3 Results

GF have significantly worse RGC survival after ONC, than SPF mice, which is reversible by conventionalization

There were no significant differences in the RGC density of SPF, GF and CON mice at baseline. At day 3 after ONC there was no significant cell death in any group. At Day 7 there were significant declines in the RGC populations of each group of mice compared to Day 0 and Day 3 (Figure 6.2). The cell survival was significantly lower in GF (40.7%) mice as compared to SPF mice and CON mice (50.4% and 48.4% respectively, $p<0.05$); this equates to a 19.2% less RGC survival in GF mice relative to SPF mice by day 7. By day 35, cell survival was even further reduced as compared to baseline, and similarly cell survival was markedly worse in GF mice (11.8%) as compared to SPF (18.1%) and CON (18.8%) mice ($p<0.05$; Figure 6.2); this equates to 34.8% worse cell survival in GF mice relative to SPF mice at day 35.

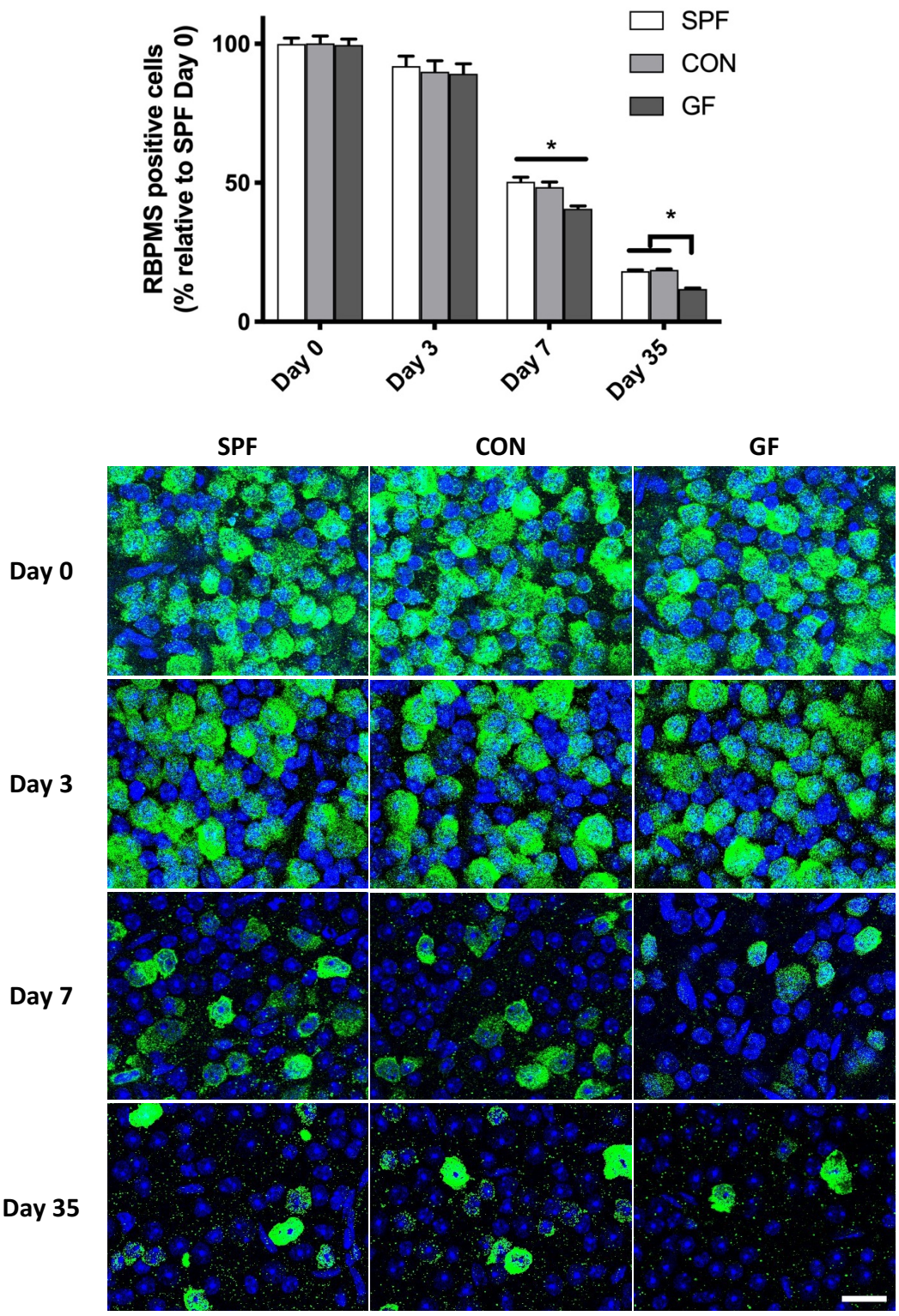


Figure 6.2: Mice with an absence of gastrointestinal microbiome have poorer retinal ganglion cell (RGC) survival after optic nerve crush (ONC)

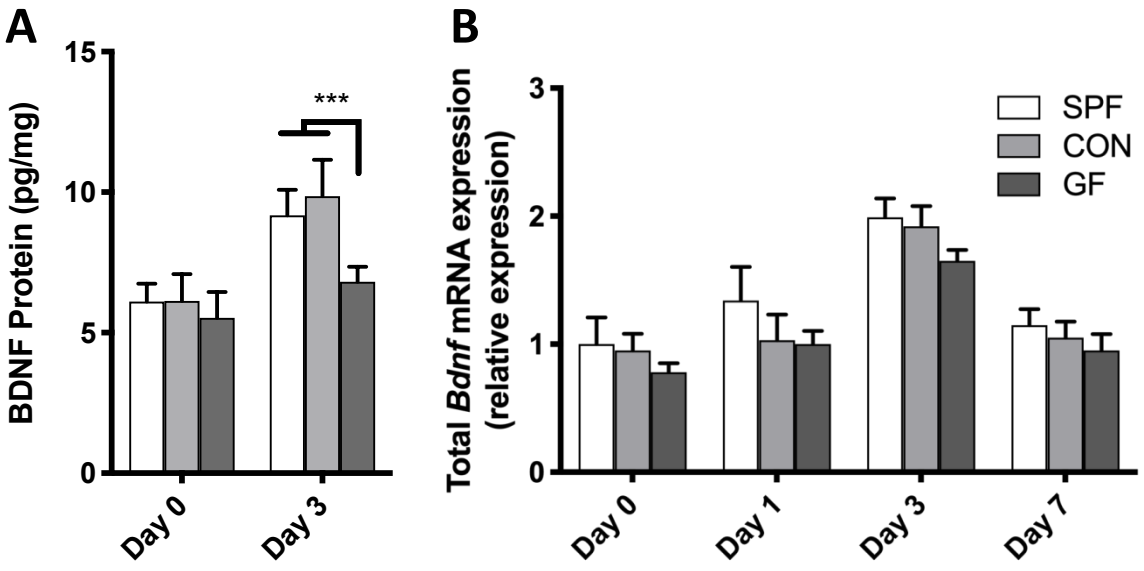
RGC cell survival after ONC calculated by cell counting of RBPMS positive cells in retinal samples from GF mice, SPF mice and CON mice. Retinal Ganglion Cells (RGCs) were stained with anti-RBPMS antibody and counterstained with DAPI. n=7-12 for all groups. Bars are presented as mean with SEM. 2-way ANOVA analysis performed, * $p<0.05$. Representative Immunohistochemistry images with green labelling RBPMS, and blue labelling DAPI are provided. Scale bar is 20μm

Protein but not mRNA Levels of BDNF are differentially altered after ONC in SPF, GF and CON mice.

At baseline there are no significant differences between the protein levels of BDNF in the retinae of GF, or CON mice, as compared to SPF (Figure 6.3A). By Day 3 after ONC, protein levels were significantly elevated compared to Day 0 in SPF and CON mice ($p<0.001$ for both) but not in GF mice ($p=0.17$). Furthermore, At Day 3, BDNF protein levels were significantly higher in SPF and CON mice compared to GF at day 3 ($p<0.001$ for both comparisons, Figure 6.3A). Indeed, GF mice had 25.8% less BDNF protein compared to SPF mice, at day 3.

Despite an increase in the *Bdnf* mRNA levels by day 3 in each group ($p<0.005$ for Day 3 compared to Day 0, in each kind of mouse), returning to levels similar to Day 0 at Day 7, at any one timepoint, the levels of *Bdnf* mRNA were not significantly different according to microbiome status (Figure 6.3B).

Figure 6.3: The presence of microbiome in mice is associated with an increase of BDNF protein expression but not mRNA, after optic nerve crush (ONC)
(A) The expression of BDNF protein, as quantified by ELISA is elevated in SPF and CON mice compared to GF mice at day 3 after ONC but not at baseline. n=6 for all groups. Bars express means with SEM. Analysis by 2-way ANOVA, ***p<0.001. (B) The expression of *Bdnf* mRNA (relative expression to *Gapdh*) in SPF, CON, and GF mice (normalised to Day 0 SPF mice) is elevated at day 3 is elevated compared to day 0 after ONC but not at baseline. n=6-12 for all groups. Bars express means with SEM. Analysis employed 2-way ANOVA. p>0.05 within each timepoint.



Injection of BDNF at the time of ONC leads to indistinguishable RGC survival between SPF and GF mice.

To determine if the BDNF neuroprotective pathway was responsible for the difference in cell survival after ONC, ONC was performed with intravitreal injections designed to saturate the BDNF receptors. In the control treated mice (those treated with the vehicle, BSA) RGC cell death proceeded similarly to what has been described previously, with GF-BSA having significantly worse outcomes by day 7 and day 35 after ONC (Figure 6.4); GF-BSA survival was 36.7% and 7.6%, respectively, compared to SPF-BSA survival of 50.3% and 14.7%, respectively ($p<0.001$ and $p<0.005$, respectively). As was expected BDNF, injections were significantly neuroprotective in both GF and SPF mice (significance at least $p<0.005$ for both GF and SPF at both timepoints, Figure 6.4). Interestingly SPF mice treated with BDNF had no significantly greater survival than GF treated with BDNF; at day 7 RGC survival of SPF-BDNF mice and GF-BDNF mice was 60.6% and 58.4%, respectively ($p=0.72$), and at day 35, cell survival was 22.4% and 19.9%, respectively ($p=0.61$).

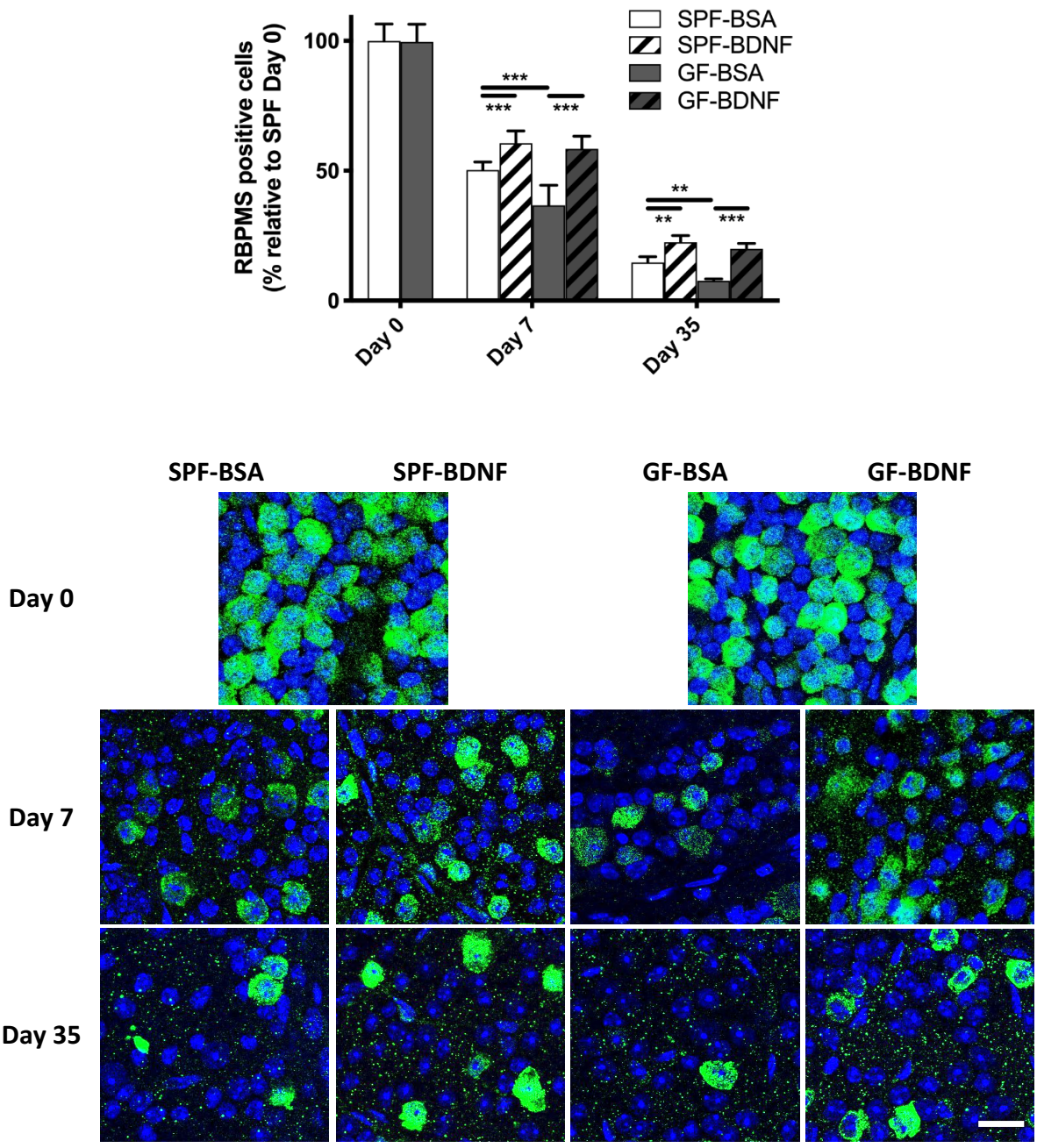


Figure 6.4: BDNF injection at the time of optic nerve crush (ONC) led to indistinguishable rates of cell survival between specific pathogen free (SPF) and germ free (GF) mice
ONC Mice given a single Intravitreal injection of BDNF protein at the time of ONC (SPF-BDNF and GF-BDNF) had significantly greater RBPMS positive RGC survival after ONC compared to mice given an injection of the vehicle at the time of ONC (SPF-BSA and GF-BSA). Analysis employed 2-way ANOVA, **p<0.005, ***p<0.001. Representative Immunohistochemistry images with Green labelling RBPMS, and Blue labelling DAPI are provided. Scale bar is 20μm

Live probiotic supplementation of GF mice increases cell survival after ONC.

L. plantarum PS128 probiotic administered to GF mice led to an increase in cell survival at day 7 and day 35 compared to GF mice, and mice gavaged with heat killed probiotic (Figure 6.5). GF RGC survival was 40.8% at day 7, insignificantly elevated in mice treated with heat-killed probiotic (41.4%), and increased to 48.6% in probiotic treated mice ($p<0.005$ for GF-Pro compared to both GF and GF-HK). Similarly, GF RGC survival was 11.8% at day 35, insignificantly elevated in GF-HK mice (12.5%), and elevated at 16.2% in GF-Pro mice ($p=0.04$, for GF-Pro compared to GF). At day 35, although GF-Pro had significantly greater cell survival than GF mice, it was not significantly greater than GF-HK ($p=0.09$).

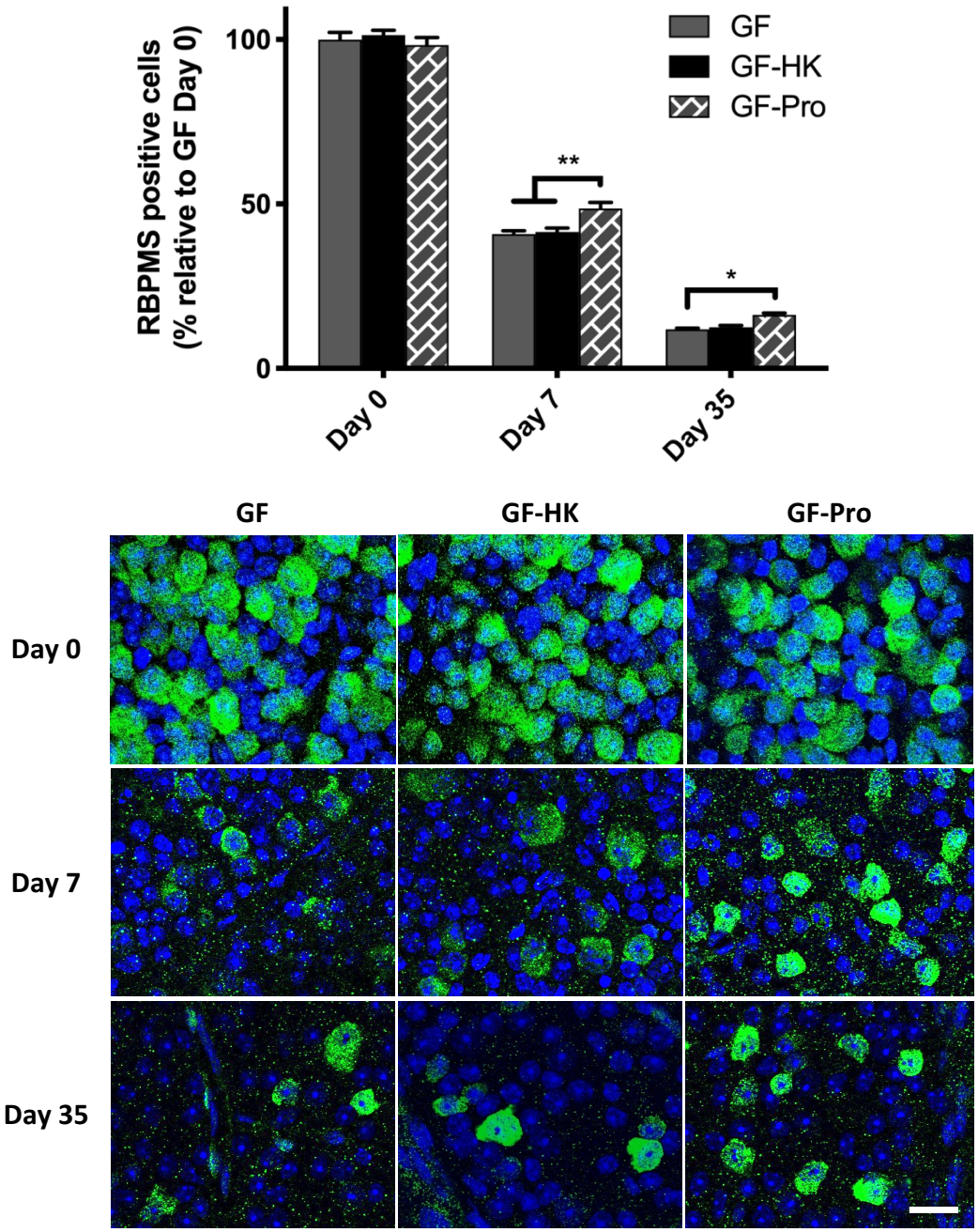


Figure 6.5: Probiotic treatment of germ free (GF) mice is associated with increased retinal ganglion cell (RGC) survival after optic nerve crush (ONC)

GF mice orally gavaged with *L. plantarum* PS128 probiotic (GF-Pro) for 14 days prior to ONC is associated with elevated RBPMS positive RGC survival and elevated BDNF expression in the mouse retina, compared to GF mice and mice treated with heat-killed probiotic (GF-HK). n=10-12 for all groups. Analysis employed 2-way ANOVA, *p<0.05, **p<0.005. Representative Immunohistochemistry images with Green labelling RBPMS, and Blue labelling DAPI are provided. Scale bar is 20μm

6.4 Discussion

The present study assessed the impact of the microbiome on an ONC model of glaucoma. From these analyses it was seen that the normal microbiome is protective to RGCs after ONC. The RGC survival of GF mice was corrected to that seen in SPF mice, by conventionalization with SPF microbiome. RGC survival of GF mice was also improved by feeding mice with live (but not heat-killed) lactobacillus probiotic.

In the literature there has been minimal research into the role of the microbiome and glaucoma however the two previous publications that have investigated this have found that the microbiome is harmful. The absence of microbiome was protective in a model of ocular hypertension. In their study, Chen *et al.* found that T cells participated in the neurodegeneration seen in glaucoma[245]. They found that the retina expressed increased levels of heat shock proteins in intraocular hypertension and that T cells activated against heat shock proteins were responsible for neurodegeneration. The microbiome contributed to neurodegeneration by priming the T cells with microbe derived heat shock proteins, thereby enacting a process of molecular mimicry[245]. Similarly, another group using a very different model found that an intraperitoneal injection of the bacterial toxin, LPS, led to worse outcomes in two ocular hypertension models[663]. The results presented in the present investigation, showing a protective effect of the microbiome, contrast with these findings, and may therefore implicate a separate mechanism in ONC as compared to intraocular hypertension. They also suggest that the results should be taken with caution and more work is required to identify the mechanisms that underly the associations seen.

BDNF protein (but not mRNA) was elevated in SPF and CON mice at day 3 after ONC. Initially it was hypothesized that alterations in BDNF expression would be observable at baseline however this was not seen in the present study. Indeed, this contrasts to the other studies of various CNS and given that the differential expression of BDNF protein occurred after the ONC was initiation, it is difficult to definitively state that the BDNF differential was responsible for cell survival differential. Despite frequent studies demonstrating that BDNF is altered in various brain regions in response to the presence of microbiome[513, 515, 516, 518], there is yet no other study demonstrating that the microbiome program the BDNF response to stimulus. Given that the BDNF differential was not present at baseline or in mRNA, further work will be required to understand the cause of the effects seen. Nevertheless, the difference in cell survival between SPF and GF mice after ONC is eliminated when both are

given injections of exogenous BDNF suggesting that when BDNF receptors are saturated, there is minimal difference in the protective mechanisms at work in SPF and GF retina. Together these results suggest that the microbiome has protective effects on the optic nerve in this ONC model through an underlying mechanism due, at least in part, to modulation of the levels of BDNF in the retina.

To begin work demonstrating that the microbiome may be a target for glaucoma therapy, and to further emphasise the beneficial aspects of the microbiome and specific components of it, a probiotic study was performed. Using the *L. plantarum* PS128 probiotic, that has previously demonstrated beneficial effects in stress responses in mice[1094], it was investigated if live probiotic supplementation would have a beneficial effect on the RGC survival in this ONC model. Live but not heat killed probiotic resulted in significant benefits to cell survival in the present model. These results indicate that individual microbes may confer benefit in optic nerve neurodegeneration. With further work it may be seen that the microbiome is a valid interventional target for further therapeutic development.

Section 4 – Discussion and Conclusions

Chapter 7 – Discussion

7.1 Summary of Core Hypothesis

Although some of the mechanisms remain to be identified and understood, there is mounting evidence that the microbiome is intricately involved in support of homeostatic mechanisms in the CNS. The hypothesis central to this thesis is that the microbiome's supportive role within the holobiont includes support of CNS systems such that abnormal microbiome may predispose an individual to glaucoma.

The comprehensive literature review outlines the documented interactions between the microbiome and the host within the holobiont and articulates how disruption of these interactions should be considered the definition of dysbiosis, a term which finds itself often flippantly applied to any minor alteration described in microbiome case-control trials. The more comprehensive definition of dysbiosis is rarely used in the literature due, in part, to the limited understanding of holobiont theory and, in part, due to expedience. IBS and dental disease are illnesses associated with microbiome abnormalities, that appear to meet the more stringent definition of dysbiosis suggested. For this reason, it was hypothesized that IBS and dental disease may be risk factors for glaucoma.

BDNF is an important neuroprotective molecule that is retrogradely transported to the retina through the optic nerve[268, 291] and also made *de novo* in the retinae of mammals[272, 284, 285, 289, 290]. It is suggested that if this production is affected that the RGC survival will be limited due to the lack of neurotrophic support. Indeed, this neuroprotective mechanism can be augmented with the use of injected exogenous BDNF and this has been demonstrated many times in the literature[340-344], and in this thesis. Similarly, it is known that the microbiome seems to regulate endogenous BDNF production. It was not known if this occurs in the retina however it is known to happen in several brain regions including the cerebral cortex[513, 515], the hippocampus[513-516] and the amygdala[515].

The hypothesis therefore became that the microbiome may help to protect the retina through support of the homeostatic mechanisms employed by the CNS. In particular it was theorised that the microbiome may regulate the levels of BDNF in the retina in such a way as to affect the propensity for an RGC to undergo apoptosis.

To determine the validity of these hypotheses a suite of research studies, involving epidemiological investigations and in experimental animal models, was undertaken.

7.2 Summary of Epidemiological Results

Two research questions comprising three research aims were posed at the beginning of this thesis, addressing the core hypothesis from an epidemiological point of view. IBS was chosen as a pathomarker for dysbiosis and was assessed in a large cross-sectional case-control study, and two very large longitudinal cohort studies. Dental health was also examined in a very large cohort study.

Research Question 1

The first hypothesis, that "Australians with advanced glaucoma will be 1.5 times as likely to also have IBS as an age and gender matched cohort of regular Australians", was addressed in Chapter 3 of this thesis. The ANZRAG cohort of people with advanced glaucoma were compared against the HCS, assessing for prevalence of IBS in these populations. This study was adequately powered to identify the hypothesized effect size (Appendix 7). Using an age and gender matched analysis, it was shown that people with glaucoma are 1.93 times as likely to also have IBS as those from the general population. Although minimal covariables were available, this proof of concept study helped to advance the research presented elsewhere in this thesis.

The second hypothesis, that "In two very large population based European cohort studies, adults with IBS will be associated with a 1.5 times increased risk of developing glaucoma over the course of the follow up of both studies", was tested in Chapter 4. In a cohort of ~9000 British individuals, from the UKBC, persistent IBS was shown to increase the risk of incident glaucoma over an 8-year period. A directionally consistent although statistically insignificant trend was seen between any IBS and incident glaucoma over this same period; though power calculations show that this cohort was underpowered to the hypothesized effect for this basic analysis (power of 0.182 for an OR of 1.5, Appendix 7).

Further evidence for the hypothesis was seen in the DNPR which found in 'time to event' analyses that physician-diagnosed IBS significantly increased the risk of an individual developing physician diagnosed glaucoma, requiring glaucoma surgery or being prescribed with glaucoma medications. These findings remained true when the follow-up period was lagged by 1 year (removing any lingering surveillance bias) and when assessed compared to cholelithiasis as a negative control (cholelithiasis is significantly less associated with dysbiosis

than IBS[1095-1097], and also presents with abdominal pain). This study was adequately powered to identify the hypothesized effect size (Appendix 7).

Because this is the first body of work, to our knowledge, that has attempted to link IBS to Glaucoma, and because the effect size was attenuated in the larger of the two studies presented, these findings should be interpreted with caution and confirmed with other studies. Although the effect sizes are relatively large and relatively consistent, further work must be performed to exclude residual confounding.

Research Question 2

The third hypothesis, that "In a large cohort of male American health professionals, people with periodontitis, or incidental tooth loss, will have 1.5 times increased odds for developing glaucoma" was examined in Chapter 5. The relationship between dental health and glaucoma has been identified previously[663], however the very large cohort presented in Chapter 5 demonstrated only a limited relationship between dental health and glaucoma. It was found that glaucoma's incidence was elevated in the 2 years proceeding from the loss of a tooth, and this was exacerbated in the context of periodontitis. This study was moderately powered to identify the hypothesized effect (Appendix 7).

7.3 Strengths and Limitations of Epidemiological Research

The advantages and limitations of a body of research must be investigated with regards to their ability to impact on the internal and external validity of the research. The hypothesis core to this suite of experiments was that IBS increases the risk of the development of glaucoma.

The internal validity of a study is its ability to address its hypothesis. It concerns itself with determining that the results seen are due to the independent variable and that an alternate explanation is unlikely to be the cause.

One of the biggest strengths of this suite of experiments is that it takes place in multiple independent cohorts that were designed by different groups with different methodologies. Although this is also particularly relevant to external validity, its relevance to the internal validity of this work cannot be understated. The multiple studies allow for greater assessment of covariables, they offer the potential for variables to be defined by different instrumentation, and likely result in different residual biasing within each study. Nevertheless,

the analyses were set up with broadly similar methodology to best determine the role of IBS in the development of glaucoma.

One of the greatest threats to internal validity in research is confounding factors. The risk factors for glaucoma and IBS have a relatively limited overlap which minimises the potential for confounding. Even so, the covariables that remain should be addressed. Accounting for covariables is an essential part of statistical modelling however for these to be addressed at the statistical level their measurement is required. As the majority of this research takes place in established cohorts, there was limited scope to add to the data collection, and so there is potential for confounding factors to have been missed. The studies analysed in this project each offered a different set of variables to be examined with some overlap and some variables unique to each study. Age and gender were available in all studies. The DNPR also included sleep apnoea, diabetes, COPD (which may be a marker of smoking), and glucocorticoid steroid usage. The UKBC also included diabetes, smoking and ethnicity. Gender and age clearly played a role in the association. The other factors did not appear to confound the effect to any significant degree, with effect sizes remaining essentially unchanged even in multiple regression analyses.

In the analysis of oral health as a risk factor for glaucoma in the HPFS, presented in Chapter 5, many more covariables were available. As this was a research project led by another group, less input was able to be given on study design. The covariables adjusted for included family history of glaucoma, race, obesity, smoking, hypertension, diabetes, physical activity, alcohol, caffeine, number of eye exams reported during follow up, cataract history, macular degeneration, and recent medical examination. Of these, it has already been discussed that smoking, alcohol, caffeine, and obesity have limited relevance to glaucoma and therefore unlikely to confound this association. Furthermore, it does not appear that family history of glaucoma or physical activity have any role in the causal pathway. Similarly, cataract history and macular degeneration are not associated with elevated risks of glaucoma, even if they do reveal the potential for surveillance bias (which is adequately addressed in the correction for by number of eye examinations, and perhaps by the number of medical examinations). For these reasons, there is perhaps an issue of ‘overadjustment' in this paper which can actually increase bias or decrease precision[1098]. Even so, the effect sizes reported in the multiply adjusted models were not substantially altered from those reported in the models adjusted only for age (Tables 5.2-5). Indeed, confounding is an issue for which

guidelines are still debated[1099]. Nevertheless, the results were not particularly convincing, in either direction, as the association was limited to the short term. Future research will be required before this link can be fully understood, and in these future analyses a nuanced approach to confounding should be taken.

On the other end of the spectrum, the ANZRAG/HCS case-control study could be assessed with scepticism given the limited covariable information. In this study, though, the E values calculated for the results presented suggests that a confounder would require an OR of 2.96 for both IBS and glaucoma to explain the effect seen, an effect size rarely seen in association studies for either illness, let alone both. Furthermore, this analysis formed a proof of concept which was assessed with greater depth in Chapter 4.

Aside from the covariables discussed in the relevant discussions in the previous chapters, SES requires some discussion. Very low income has been seen to be a risk factor for glaucoma in a number of studies[113, 166], however, in a Taiwanese registry study, income had a positive correlation with glaucoma[165], and in the Rotterdam study, no association between income and glaucoma was seen[125]. Level of education is often another instrument used to evaluate SES. Again there have been conflicting results with some articles demonstrating no effect[113, 163], others findings a positive association[164, 167]. In the Beijing Eye study, where all participants underwent a detailed ophthalmic examination, which is arguably the best method for glaucoma identification, level of education was not associated with open angle glaucoma, despite being associated with higher myopic refraction, and lower rates of cataract angle closure glaucoma[163], suggesting that education interacted with pathways associated with other ocular diseases but not open angle glaucoma. On this line, urban living has been suggested to be associated with glaucoma. In a global review of glaucoma prevalence, studies in urban populations had higher rates of glaucoma[17]. Even so, there have been several studies that have not demonstrated this association including from China[834], Nigeria[169] and Australia[170].

The suggestion that IBS and SES may be related seems to come from the supposition that IBS is associated with urbanisation and national development. These theories seem to find their footing in the growing prevalence of IBS in Asian countries[706, 835]. Even so, one study that looked directly at this effect found no association between the Human Development Index of a nation and IBS, and virtually no difference between the developing and the developed world except for methods of data collection[1100]. Also, it was reported in a

Singaporean cohort that IBS may be associated with increased western food intake perhaps indicating a dietary effect rather than an urbanisation effect in IBS[1101]. Other studies have determined that affluence in childhood, specifically, may be related to IBS, finding that IBS was associated with a lower living density (<1 person per room)[1102] and higher social class (as defined by parental occupation)[1103] during childhood. These findings have perpetuated the hygiene hypothesis in IBS[811]. Education level does not appear to play a role in IBS prevalence[1104-1106]. An online survey-based study of almost 26000 people found that lower income was associated with IBS[1107]. In a Colombian study of university students, IBS was noted to be higher in individuals with lower socioeconomic status (as determined by income level)[1108]. A similar study in Lebanese university students showed the opposite effect with high income predicting IBS[1109]. Furthermore, socioeconomic status in adults was meta-analysed in 2012 and found to have no effect[699].

When assessing the role of SES in IBS and glaucoma, neither appear strongly related to SES status. Education status seems to be only weakly associated with glaucoma, and as it is not associated with IBS, therefore it is unlikely to be able to confound the IBS-glaucoma relationship. The likelihood that income, which also has conflicting findings with IBS and glaucoma, is a confounder is similarly unlikely. Finally, although urban living has been suggested as a risk factor for IBS, based on national level data[706, 835], the findings that development index has no effects on IBS prevalence makes this unlikely. As each of the studies in this thesis are national studies the study populations include both rural and urban communities and therefore, if urbanisation could confound the findings, there is an opportunity for this to be occurring in the presented research. Future research will require consideration of this factor to further assess these associations.

These cohorts and methodologies were chosen in a way to minimise potential biases however it is worth analysing from where sources of bias may have come. Beyond confounding, systemic bias mainly falls into two broad categories, information bias and selection bias[1110].

Selection bias occurs when the study cohort does not accurately represent the target population as indicated in the hypothesis[1110]. The hypothesis being tested here applies to all adults, and therefore the study population should emulate the adult population.

The UKBC is a birth-cohort that attempted to capture every child born in the UK in a single week of 1958 (with 98.7% rate acceptance), with every immigrant child born in the

reference week added to the target sample throughout schooling years. The total cohort size is 18558 individuals including all perinatal deaths, and all immigrants, although the largest sweep occurred at birth, with 17415 participants. During school, the participants were tracked through the schooling system, so at the first adult sweep (age 23) the sample was considerably smaller than the sample reached at age 16, with only 76% responding at age 23 compared to 87% participation seen at age 16. The largest source of attrition is participants changing addresses or not responding to investigators however a small proportion of participants ~10% refuse to participate at any given sweep[1040]. The most significant potential sources of selection bias in this cohort are attrition prior to data collection.

At age 42, 11419 participated of a total potential 16091 (living and resident in the UK). Refusal accounted for 1148 of the non-responders with the remaining non-response due to loss of contact. At age 50, only 9790, or 62%, participants responded of a total possible cohort of 15806[1039]. The total cohort that participated in both sweeps was 9092. As this study essentially begins at the age 42 sweep, attrition until this point essentially acts as the non-response rate. People who died or emigrated, for obvious reasons, cannot count in the total population and therefore the potential response number at age 42 was 16091, of which the achieved response rate was 71.0%[1039]. The response rate at age 50 was 9790, 60.8% of the total possible cohort at age 42, with death and emigration responsible for 285 of this loss to follow-up and the remainder due to continued or new nonresponse[1039]. Of those 11419 that had participated at age 42, 2327 (20.4%) did not participate at age 50[1039]. Incidentally, a 20% drop-off between sweeps of a cohort study is not unusual[1111] (although it was comparably large for this specific study[1039]). Similarly these response rates are comparable to other large cohort studies; the Blue Mountains Eye study had a response rate of 82.4%[4], the Framingham heart study had a response rate of 68%[1112], The Australian Longitudinal Study on Women's Health had a response rate of 38.1%[1113], however large studies have seen response rates as low as the 5.5% response rate in the UK Biobank study[1114], or 24% in the second Nurses Health Study[1111].

IBS was identified in 959 (8.6%) of UKBC participants at age 42; however, 181 (18.8%) of these did not participate at age 50. IBS sufferers were similarly likely as non-sufferers at age 42 to participate in the sweep at age 50 (p=0.23). Similarly, glaucoma sufferers at age 50 were equally likely to have participated at age 42 as non-sufferers (p=0.63). From these factors it does not appear that IBS affects subsequent attrition, nor does a glaucoma diagnosis

affect participation. Although neither the exposure nor the outcome variable was associated with attrition, females who responded at age 42 were 23% more likely than men to also respond at age 50 ($p<0.01$).

There is another aspect of potential selection bias even in this sample as it does not account for immigration beyond school years. Given that the hypothesis in question in relation to the total adult population, the ideal study would include a complete cross section of the adult population. The birth cohort effectively captures the diversity of the population at its initiation however with attrition, and by omitting immigrants beyond school years, there remains potential for the sample to be skewed once the participants have reached adulthood. If the group incompletely represented in the sample offer a different IBS-glaucoma relationship, bias would result. It seems unlikely that immigration status or attrition would affect this association, however, this cannot be ruled out.

The DNPR is drawn from the total population of Denmark and therefore should be population representative. There is a minor role for healthcare access bias in this study; however, as Danish healthcare services are socialised, it would seem unlikely that patients wouldn't be able to attend healthcare for purposes of being unable to afford it[1115]. The sources of selection bias in the DNPR come mainly from the spectrum bias that is associated with defining IBS by hospital records. The DNPR collects data from all hospital contact including within the National Health Service, Denmark's nationalised healthcare system, since 1977 and includes hospital outpatients visits since 1995[1116]. This data includes both the primary diagnosis and also comorbid diagnoses. The prevalence of IBS in the DNPR is 0.85%, which is considerably lower than the UKBC (8.6% of participants at age 42) and other reported studies[706]. This low prevalence is likely because the management of IBS is largely the domain of general practitioners, and therefore a good proportion of people with IBS may never mention it within a hospital setting. For this reason, it is also possible that the IBS cohort in this study is likely to be made up of more severe IBS, hence requiring hospital care, rather than just regular patients who mention it, only for it to be added to their 'other diagnoses' list. If the effect of IBS on glaucoma is real, then this could arguably bias the results in either direction. Firstly, given that it is possible these IBS patients represent a more severe diseased group, and it is possible that the severity of IBS may correlate to the severity of the underlying dysbiosis, then potentially limiting the IBS cohort to just the severe cases may accentuate the effect. Conversely, as approximately only 10% of the total IBS cohort was identified, the

remaining ~90% of the people with IBS in the cohort remain in the control group (comprising up to 9% of the control cohort), and therefore if the effect is real these would artificially boost the rate in the control population biasing the effect toward the null. These considerations are important when evaluating the clinical significance of a statistically significant finding. As this factor acts as both selection bias and information bias, this will be discussed further in the Information bias section. This study also used matching within the cohort to control for age and gender which have been argued to be potential confounding factors however matching has potential to bias studies (toward the null) if it is 'overmatched', by a factor that is associated only with the exposure variable[1110]. Finally, as DNPR is a registry study that uses data provided by hospitals to the national databases with no opt-out mechanism, there is virtually no chance for loss to follow-up, participant withdrawal or non-response, as all data is automatically captured[1116].

Finally, the ANZRAG case-control study suffers from both spectrum bias and also potentially matching bias. ANZRAG is a cohort of Australians with advanced glaucoma, designed for use in genetics studies, that is generally made up of patients with often more aggressive glaucoma phenotypes and as such the cohort isn't necessarily representative of the spectrum of glaucoma, indeed it has been shown that the myocilin gene mutation is more prevalent in this cohort than in less advanced cases[1117]. The study, like the DNPR, is matched on age and gender and therefore if either of these is not a true confounder, then the effect size may also be minimised by over matching[1110]. As the ANZRAG cohort is being compared to the HCS cohort, this cohort's selection bias should also be addressed. The HCS is a population representative sample with almost perfect demographic parity to the Hunter region that it is drawn from (which was chosen due to its broad similarities to the Australian national demographic breakdown)[1033]. The response rate in the HCS was high at 77.4% with insignificant differences in the responders, to the total source population and broader national populations in either gender or marital status, despite being slightly younger[1033]. The age limitation may come from the exclusion criteria of living in residential care, which is unlikely to bias the results of the present study.

Detection bias is usually a form of information bias however it can work as a selection bias in case-control studies if the exposure of interest can impact on the diagnosis of the disease outcome[1110]. It seems unlikely that IBS would impact on the diagnosis of glaucoma. In the ANZRAG trial, glaucoma has already been diagnosed, and IBS is determined by survey

results, so IBS here has virtually no ability to impact on glaucoma detection. Detection bias in the UKBC and the DNPR is discussed further with information biases.

Information biases occur at the point of data collection and are due mainly due to measurement error. Information bias may occur due to misclassification, detection or recall errors. One of the most significant impacts on internal validity comes in at this point, namely the choice of instrumentation.

The UKBC is a robust study with regards to its breadth of data however the data comes mostly (and entirely, for the purposes of this thesis) from self-reported variables. There is justified scepticism in the literature regarding the legitimacy of self-reported data variables. In the cancer literature, for example, it has been shown that self-reported diagnoses of cancer suffered low sensitivity and that alarmingly the sensitivity was lowest in smokers, whilst highest in the educated population, which clearly may significantly bias results[1118]. That said, Inflammatory Bowel Disease has been shown to have strikingly high agreement between self-reported measures and disease history which may potentially translate to IBS[1119]. The IBS and glaucoma measures identified within the UKBC have undergone no validation, and therefore caution must be taken when relying solely on these results.

The IBS prevalence in this cohort is 8.6% which is within the normal of range (6.1%-21.68%) of what has been reported previously within the UK, although on the lower end of the scale[706]. The relatively higher rates in other prevalence studies are likely due, in part, to prevalence estimates coming from diagnostic instruments being used to identify IBS patients rather than asking about diagnosis, and therefore includes a proportion of the community who would not have otherwise been diagnosed. Without the inclusion of a diagnostic instrument in the survey, a self-report of IBS implies a physician diagnosis and therefore should exclude the undiagnosed IBS sufferers. This is reinforced by the studies that have shown that the rate of people meeting the criteria for IBS in the population is often markedly higher than those who have received a diagnosis[1120, 1121].

As the data is collected at specific time points with questions regarding the period since the previous sweep, there is also the potential for recall bias in this study. Glaucoma is an illness often with poor adherence[1122] and low symptom burden, for this reason, it is reasonable that a diagnosis of glaucoma could be forgotten by the time of a follow-up survey. IBS patients have been noted to have minor but significant verbal and visuospatial memory defects[1123-1125], which may be due to the psychology associated with IBS. Perhaps these

deficits could interfere with these participants remembering a glaucoma diagnosis in a way that may minimise the effects size. The rate of glaucoma at age 50 is 0.58% which is within the expected range of 0.36%-0.89% expected in white populations for this age-group, based on a meta-analysis of large population-based studies in Europe, Australia and the USA[1126]. Given that this rate lines up well with the expected range of prevalence, misclassification within this group may be less frequent. Similarly, as glaucoma is an illness, with relatively poor awareness, in the general community[1127, 1128], it is unlikely that spurious false positives would be captured.

Surveillance bias is minimised in the UKBC simply by design of the study questionnaires. These questionnaires take a systems-based approach to the diagnosis of comorbidities and allow the participant to think about each potential system including issues with eyes and issues with bowels or abdominal pain. The UKBC is unlikely to suffer from differential surveillance, and the style employed in its questionnaires may lower the incidence of false negatives. Also, the UKBC allowed for the creation of a 'chronic IBS' variable which showed a more severe interaction between IBS and glaucoma, which fits biologically with expectations.

The DNPR is the study with the largest likelihood of misclassification bias, and there also may be potential for detection bias. As has already been emphasised, the hospital registries from which the cohort was drawn include approximately 10% of the IBS prevalence seen in the UKBC. In fact, within the Danish population, IBS prevalence was estimated to be 16%[1129], so it is possible that the captured IBS rate (as a proportion of its true prevalence in the population) is lower than this. Although this is a weakness of our study, this misclassification bias, would likely bias the effect toward the null. However, if it is that the misclassification is non-differential (i.e. that the IBS cases represent more severe cases), then the misclassification would not be considered non-differential and may increase the effect size. Nevertheless, for this to occur the underlying hypothesis would still be correct (i.e. that IBS and its dysbiosis are associated with glaucoma) however the effect size would be inaccurate. The fact that the effect size appears attenuated in this study compared to the other studies suggests that perhaps misclassification is biasing toward the null.

To combat any detection bias in the definition of glaucoma in the DNPR, three separate definitions were chosen for glaucoma. These definitions required input from different parts of the DNPR including linkage to the Danish National Prescription Registry. The

first definition, physician diagnosis of glaucoma, was acquired from ICD-10 codes in medical contact summaries in a similar way to IBS. Under-detection is likely in this mechanism as general practitioner, optometrist and private ophthalmologist consultations are not included in this database. Glaucoma surgery was sourced from surgical data in the DNPR. Misclassification is minimised in this definition as all surgical operations performed are required to be entered into the database. Similarly, the medication definition suffers from limited misclassification in that all prescriptions are managed through nationalised single payer systems, and so virtually all prescriptions are covered by this database. These additional definitions of glaucoma are superior in their sensitivity than the diagnostic coding however as they only define specific cohorts under the umbrella of glaucoma, their validity is more limited, and they are probably better suited to 'checking' the physician diagnosed definition than standing on their own. For example, only a small segment of glaucoma patients ever require surgery, and generally speaking, it is only offered if the ocular hypertension is refractory to medication. Although, naturally, surgical patients will skew more severe in their glaucoma phenotype, there are many patients who have severe glaucoma despite having low pressures, and therefore this isn't simply a definition of severe glaucoma. On the other hand, the medication definition of glaucoma includes people who haven't strictly been diagnosed with glaucoma[1130]. Many patients on glaucoma medications suffer only with ocular hypertension and are simply being given these medications as a precaution. The consistency of the results across these definitions is a considerable strength as, despite their significantly different methods for being defined as cases, they all are similarly impacted on by IBS.

The DNPR also provided a suitable negative control in cholelithiasis. Cholelithiasis is another illness that presents with abdominal pain however as it is due to a clear pathology that is essentially unrelated to IBS, it should be useful to determine if the associations between IBS and glaucoma are representative of some bias within the study that inappropriately links abdominal pain illnesses with glaucoma. There is also only limited microbiome effects in Cholelithiasis[1096, 1131] suggesting that this also acts a suitable control even when considering IBS as a pathomarker. The consistency of the results seen in this control group are another strength of this study.

The ANZRAG/HCS study has the greatest risk for bias of the studies chosen however also gave the greatest opportunity to address these biases. Firstly, the information bias that can occur in the choice of an instrument for the analysis of a variable is one source of bias

that could have occurred, which was addressed by using the ROME criteria rather than asking about previous diagnoses or requesting physician reports. This methodology ensures that the diagnosis of IBS is at the highest standard within this cohort compared to the other cohorts tested, and the rate of IBS being significantly higher in this cohort than other suggests that this method captures a significant burden of undiagnosed disease in the community which in other studies would be misclassified as healthy controls. There is something to be said for the fact that the population-based cohort, HCS, and the glaucoma based cohort, ANZRAG, were recruited through substantially different methods. Given that ANZRAG is voluntary registry of physician referred patients, and the HCS is population-based cohort of community members, the expectations of the two groups may vary when it comes to answering surveys. Similarly, the IBS questionnaire was part of a larger questionnaire for the HCS participants compared to the ANZRAG population, who received a smaller questionnaire to maximise the likelihood of returning the survey, and also limit the collection of irrelevant data. Nevertheless, these issues seem more likely to affect selection bias (i.e. the answers to the questions themselves are unlikely to be altered by these differing circumstances, but perhaps one's likelihood of returning the survey might be). The strengths of this study are that glaucoma and IBS are extremely well categorised, however the weaknesses are those typical of case control studies where it is difficult to determine how representative of 'cases' in the general population the cases within the study are.

The strengths in this suite of studies are particularly evident the external validity of the collected work. These studies demonstrate a directionally consistent association between IBS and Glaucoma. Although the effect sizes are different in the studies, these may be explainable by the differing methodology in each study, particularly with regards to the classification of IBS which has significant different rates across the studies.

Three studies addressing the same question in separate cohorts significantly strengthens confidence in findings. Each of these studies is a large cohort of patients based in a first-world nation with good access to quality healthcare, limiting falsely identified cases of either IBS or glaucoma. As definitions of cases are reliable, there is comparatively limited opportunity for spurious correlations to be identified. For this reason, these findings should be applicable to largely white populations in the developed world. Based on the literature regarding ethnicity and its relationship to IBS and glaucoma, there is little to suggest that these findings would not similarly translate to a cohort of a different ethnicity (although in

nations with lower access to healthcare, considerations would be required in the data collection and analysis).

The association between gastrointestinal health and glaucoma is one that has not, to our knowledge, been explored previously. However, this result is consistent with previous work linking IBS to other neurodegenerative illnesses including both PD[926] and AD[570]. As PD, AD and glaucoma all have different aetiologies and natural histories, it seems that IBS has a universally negative impact on CNS homeostasis particularly with regards to neurodegenerative disease. Given that IBS shares minimal risk factors with these illnesses, and virtually no symptomatic overlap, except for constipation seen in PD[1132, 1133], it is likely that some component of IBS pathology compromises CNS homeostasis predisposing these individuals to neurodegeneration. I propose that the association between IBS and Glaucoma is due to microbiome host-mediated effects.

7.4 IBS as a Pathomarker for Dysbiosis

As has been extensively discussed in '1.5.1 A Pathomarker for Dysbiosis', there is a good breadth of data to support the use of IBS as a marker for dysbiosis in epidemiological investigations. Even so, there are few points worth discussing regarding its use.

Firstly, it is important to recognise that the microbiome likely differs between IBS subtypes. A 2019 systematic review found that five of ten studies comparing subtypes noted differences between IBS-C and IBS-D, however the differing taxa reported in these articles did not form a pattern[724]. As the microbiome associated with each IBS subtype may be different from each other, these differences merit consideration. If the microbiome abnormalities associated with one subtype are specifically responsible for the effects seen in the documented associations between IBS and glaucoma, then this likely dilutes the effect size seen, which is a limitation of the research presented in this thesis. Future studies will certainly need to dissect which IBS subtypes primarily contribute to these findings.

Secondly, one needs to consider the alternate reasons for a relationship between IBS and glaucoma. Although the microbiome's association with IBS is what initiated this investigation, there are a number of alternate biologically plausible pathways that may explain the observed association between IBS and glaucoma. This may affect the external validity of these results for broader microbiome interpretation. If the effects of the

microbiome on glaucoma are due to factors outside of the microbiome, these results may only be externally valid to other populations of people with IBS.

Beyond the microbiome, the next most plausible mechanism that may explain the association between IBS and Glaucoma is alterations in the immune system. Although IBS is an illness with a limited level of host pathology, there has been some small alterations in the immune systems of people with IBS[786, 787] which may also play a role in glaucomatous pathology. There has been some indication that people with IBS may have a mild elevation of certain inflammatory markers such as IL-6[787], IL-8[787], TNFα[1134] and IL-10[1134]. That said, one study of a large panel of circulating cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα) found no differences between IBS patients and healthy controls, finding that only a single local colonic effect was significant (namely reduced colonic IL-10 mRNA was seen in female IBS patients)[788]. A meta-analysis of IL-10 and TNFα suggested that circulating TNFα was marginally elevated in female patients, however when men were included the finding was insignificant[1134]. This meta-analysis showed no significant effects in IL-10 except for validation of the result by Chang *et al.*[788], that local mRNA levels were reduced in the IBS colon[1134]. In one study, isolated peripheral blood mononuclear cells were shown to be hyper-reactive with upregulation of inflammatory cytokines compared to those from healthy controls[786]. For these reasons, there is a general consensus that low-grade inflammation *may* play a role in some IBS[1051] and presents another potential mechanism that could link IBS and Glaucoma.

Although cytokine elevation in IBS appears to be a weak finding, with conflicting literature published on the subject, the concept merits some thought when addressing the cause of the link between IBS and glaucoma. Traditionally molecules the size of cytokines which typically range from 5-20kDa were thought to be too large to cross the blood brain barrier[1135], however since the initial discovery of IL-1α transport across the BBB in the late 1980's[1136] (and subsequently validated in the early 2000s[1137]) transport mechanisms for many cytokines including the relevant IL-6 and TNFα have been discovered[1135, 1138, 1139]. These findings have been replicated in other animals[1140] suggesting that this mechanism is likely present in humans. The activity of these cytokines once passed to the central nervous system is unclear. TNFα is a cytokine that acts on the TNFR1 and TNFR2 receptors to achieve apoptosis[210] with documented evidence of activity in the retina. The direct administration of TNFα to the retina leads to RGC death without any other insult, similar to what is seen in

glaucoma models[213]. Deletion of its receptors TNFR1 and TNFR2 offer protection in glaucoma models[213, 214]. However, the role of TNFα is not clear in glaucoma, with treatment of retinae with TNFα prior to an ONC demonstrating a protective effect for RGCs[1141].

The role of IL-6 is highly circumstantial with both pro-inflammatory and anti-inflammatory effects[1142]. The activity of IL6 is not clear in glaucoma; there is evidence that this cytokine may play an important protective role in axonal damage[207, 1053, 1143], and may also play protective roles elsewhere in CNS illness[1144]. The other cytokine which may be relevant to IBS, IL-8, which was shown to be elevated in IBS by Dinen *et al.*[787] but not Chang *et al.*[788], has been shown to have cytotoxic effects in cultured neurons[1145], however, there is limited literature assessing its potential for initiating ganglion cell degeneration.

Hyperactive peripheral blood mononuclear cells have the theoretical potential to be involved in CNS damage. Although these cells usually stay out of the CNS, their translocation across the blood brain barrier is possible and has been noted in response to certain CNS insults[1146-1148]. For this reason, it is conceivable that slightly more aggressive peripheral blood mononuclear cells, such as those described by Liebregts *et al.*[786], may exert a somewhat more aggressive inflammatory response in the retina if they were to translocate into the retina.

The discussion regarding the possibility of low-grade inflammation playing a role in glaucoma's pathogenesis, specifically, has been controversial[1035]. Despite the inflammatory changes that may occur in experimental glaucoma, there is only a little evidence in humans that there is a systemically detectable inflammatory effect. Even so, there has been some suggestion that certain inflammatory cytokines, IL-4 and IL-6, may be elevated in the serum of people with glaucoma[1052], in this study, other cytokines, IL-2, IL-12p40, IL-12p70, IL-23, TNFα and INFγ were equivocal between the glaucoma and healthy cohorts[1052]. Moreover, despite its elevation in that study, IL-6 has been shown to be lower in the aqueous humour[204, 1149, 1150], and tear films[202], of glaucomatous patients. These findings are interesting given the dual pro and anti-inflammatory effects that IL-6 may have. Other articles, however, have found its presence no different between glaucomatous and non-glaucomatous eyes[198]. IL-8, which is unlikely to play a protective role, has been confirmed to be elevated in the aqueous humour[198, 1149]. Interestingly the surgical burden, experienced by an eye, appears to have significantly greater effects on the cytokine profile of the eye than glaucoma disease status[1150]. Also as it appears that vitreous cytokine levels are not differentially expressed in glaucomatous eyes (except in acute angle closure glaucoma)[1151], it's possible that the

cytokine profile of the aqueous humour and the tear film is representative of anterior chamber inflammation associated glaucomatous changes or ocular surface inflammation associated with drop usage. In fact, chronic drop usage has been associated with inflammatory cytokine production in the conjunctiva[1152, 1153], and in the aqueous[200] of glaucoma patients. Moreover, it is known that the corneal epithelium readily produces significant quantities of IL-8 in response to relatively minor stimulation[1154]. Determining the role of inflammation in the link between IBS and glaucoma is likely to be more difficult than analysing the microbiomes role, however, with well-designed animal and human studies, this is an avenue that should be explored.

The image of the neurotic IBS patient is one that has developed not without cause[1155]. The question then becomes if people with IBS are over utilising healthcare to the point where diagnoses of illnesses unrelated to IBS are being made simply by elevated surveillance. It is well known in the literature that IBS patients have greater medical contact than their 'healthy' counterparts[1156, 1157], however it seems that these patients seek care for pain rather than due to increased neuroticism[1157, 1158]. In one study, pain factors alone and not psychological factors were associated with seeking medical care in IBS patients[1158], suggesting that consultation is more often in regards to their disease and less simply due to spurious reasons. Another study found that, while IBS patients were significantly more likely to use antibiotics than healthy people, other drug classes were not significantly elevated in IBS patients[1102] making medication effects an unlikely cause of the effects seen. IBS triggered increased healthcare contact is therefore unlikely to be associated with elevated glaucoma diagnosis, especially when the ocular domain of healthcare is handled by optometrists and ophthalmologists, and rarely ever handled by general practitioners.

7.5 Implications of Epidemiological Research

The practice of epidemiology is responsible for some of the most important medical discoveries in history. John Snow, renowned as the father of epidemiology, is well known specifically for his epidemiological research that tracked down a London cholera outbreak to a specific well located near a cesspit in 1854[1159]. This finding, amongst others (even prior to this), were used to establish his theories regarding the faecal-oral route of disease transmission, even ten years prior to the work of Louis Pasteur[1160]. He further postulated, at a time where the miasma theory of illness was dominant, based on his findings, that a cell,

capable of replicating in the bowel, was responsible for cholera[1160]. This work is demonstrative of the power of epidemiology for the investigation of deep biological questions. Similarly, this project uses epidemiological methods directed at biological hypotheses to investigate the extent of the interactions within the holobiont.

This study has several important indications for clinicians and other researchers. Firstly, ophthalmologists (and optometrists involved in the management of glaucoma), gastroenterologists, and general practitioners (who provide the majority of care to people with IBS) should be aware of the potential for increased risk of glaucoma in people with IBS. Given that glaucoma is relatively easy to treat and also given that it is progressively blinding and currently irreversible, it behoves the careful clinician to have an extra level of suspicion of glaucoma in IBS patients. Secondly, although this research does not indicate any variation in the care of glaucoma patients with IBS, future research should address the two potential mechanisms discussed above. Careful investigation of the role of microbiome dysbiosis and immune system dysregulation in glaucoma's pathogenesis may lead to new therapeutic options for glaucoma patients. Thirdly, these results should also be considered in light of other epidemiological reports indicating that IBS patients are at an elevated risk for the development of neurodegenerative illnesses[570, 926]. These findings may prompt researchers investigating CNS homeostasis to address broader mechanisms of IBS and how they may be related to neurodegenerative pathology. Thirdly, although the majority of glaucoma is POAG, this study is unable to determine the subtypes of glaucoma most at risk in IBS patients, future research must address the phenotypes of Glaucoma that are most typically associated with IBS. Finally, the two cohorts presented demonstrated differing effect sizes for the association between IBS and Glaucoma indicating the need for follow up study of this phenomenon. Although the disparity may be due to the under-reporting of IBS in the DNPR or some unrevealed bias in the UKBC, understanding the true effect is important for informing the decision making of clinicians in this field.

7.7 Summary of Animal Model Results

The animal research presented in this thesis addressed two research aims, as outlined in '1.7 Aims and Hypotheses', both research aims were addressed in Chapter 6 of this thesis.

The first hypothesis related to animal research was that “By day’s 7 and 35, after ONC, GF mice will have 20% less RGC cell survival relative to SPF mice at the same timepoint, and

that CON mice will have similar cell survival to SPF mice". In an ONC model performed on GF, and SPF, it was shown that the absence of microbiome had a significant adverse effect on the survival of RGCs after ONC, with GF mice having 19.2% and 34.8% less RGC survival relative to SPF mice at day 7 and 35, respectively. Conventionalisation of GF mice led to similar protection of the RGCs. Treatment with a probiotic treatment of *L. plantarum* PS128 for 2 weeks prior to crush procedure and after procedure until harvesting, also had a protective effect. Heat Killed probiotic did not have this effect.

The second main hypothesis guiding the animal research presented in this thesis was that "GF retinae will have 30% less BDNF than the retinae of SPF and CON mice, and that this difference will increase further by day 3 after ONC". In the research presented, at day 3 but not at baseline, GF retinae had 25.8% less BDNF protein than SPF mice. *Bdnf* mRNA was not differentially expressed in any measured timepoint after ONC.

Intravitreal injection of BDNF had a significant protective effect in both GF and SPF mice, and the RGC survival in eyes injected with BDNF were indistinguishable by 'microbiome status'. Further work addressing the expression patterns of BDNF and its regulation is planned.

7.8 Strengths and Weaknesses of the Animal Research

The animal research presented in this thesis examined the hypothesis that the microbiome plays a neuroprotective role in an ONC model of glaucoma. Just as with epidemiological research, the strength of the research must be weighed on the grounds of its internal and external validity.

Although animal research allows for elimination of many of the biases typically seen in epidemiological research there are still threats to internal validity that must be discussed.

The use of GF mice introduces considerable potential for selection bias in these studies. Randomization is not possible in this type of study as the establishment of GF status requires multiple generations of mice. Similarly, blinding is difficult at most stages of experimentation as there are gross phenotypic differences between germ free and SPF mice, namely the abdominal size due to caecum size[887]. This significantly noticeable feature means that mice even if mice are shielded from the researchers view until the time of the ONC, the procedure cannot be well blinded. For the same reason, the tissue harvest process is difficult to blind. Tissue sample preparation and sample analysis was blinded with numbers given to each

sample, and these matched back to mouse condition only once processing and analysis on each sample had been done.

Given that randomization is not possible, whole litters of mice were used with no exclusions from the litters provided. Furthermore, the majority of the animal husbandry was performed by vets and technical staff employed by the animal housing facility, and therefore was independent of the experimentation.

In the preliminary interventional components of this research, randomization and group allocation was performed independently by the veterinary and technical staff. The technical staff were asked for a certain number of mice that were required for any particular interventional experiment, and the number of groups required, (the intervention details were not discussed) and they independently, without researcher input, separated cohorts of mice for these experiments as requested. The only deviation to this protocol was for the probiotic treatment which required the contamination of a GF insulator with probiotic bacteria and in this case the randomization and allocation was performed by the independent technical staff however treatment allocation concealment was not possible. Although not the most rigorous of randomization or allocation concealment, it is probably sufficient for this preliminary phase of research in this area.

Randomization and blinding remain two significant issues with a majority of animal research. Hirst *et al.* assessed these issues in their review paper, finding that of 31 systematic reviews of interventional animal research (in "any" disease area and outcome) only 29% of studies reported randomization, and 35% of studies reported blinded outcome assessment[1161]. Reassuringly, in their analysis they found that articles that reported blinding did not have significantly different effect sizes (p=0.67) however randomization led to smaller effect sizes, with a standardized mean difference of -0.07 (p=0.008)[1161]. Although the effects, at a meta-regression level, are small, the potential for these to affect internal validity are the principal reasons why their control has become standard practice in human interventional research[1162]. Over the past 20 years researchers investigating human disease have pointed out notable failures of translation from animal studies[1163-1166], including opposite effects noted in human trials of certain interventions compared to animal studies[1163], the majority of these reports cite issues with randomization and blinding as core to the issues with animal research. The relevance of these cautions is somewhat diminished by the observational

nature of the major elements of this research. Further interventional research will require a stricter approach to these factors.

Adequate controls are also important to identify if processes that occur within the generation of a model or the analysis of results could bias the effects. One important process that may have considerable effects on the mice that may be completely unrelated to their microbiome status is the process of deriving a GF animal, and the subsequent considerations in their housing. This is controlled for by comparing a third group of mice, the CON mice. GF mice were re-conventionalized within the GF insulators with microbiome from SPF mice to determine if the GF derivation and housing processes, specifically, had effects beyond the microbiome. In this research, the CON mice had very similar outcomes to the SPF mice suggesting that there were minimal impacts caused by the techniques required for the maintenance of GF status. This minimizes suspicion of this potential threat to internal validity.

Experimental mortality (the only reasonable cause of attrition) is also potential sources of bias, although this was not an issue within this research.

There has been a lot written on the external validity of mouse models for the research of human diseases. Indeed, as discussed above there is some suggestion that the failures of translation from animals to human disease may come from issues that primarily affect internal validity. Even so, the external validity of (internally valid) animal research requires consideration. The similarities and differences between humans and mice should therefore be carefully evaluated.

The genetics of a mouse are significantly different to humans. The differing rates of genome homology (depending on the definition used for homology[1167, 1168]) may or may not take into account the significant differences in gene regulation that can occur between the species. For the purposes of this argument, the *BDNF* gene will be taken as an example. *BDNF* has the most complex genetic structure of the neurotrophin family. Mouse *Bdnf* is complex with 11 transcript variants, stemming from eight non-coding exons and one protein coding exon,[1169] however the complexity of this gene is far superseded by the human *BDNF* gene for which 17 transcript variants have been identified[1170]. To further complicate human BDNF genetics, an antisense *BDNF* is also produced at the same site which itself has 12 transcript variants[1170], compared to the two seen in mice[1171]. Even though this gene would be counted amongst the 80% of mouse genes that have a single human orthologue[1167], it is clear that the regulation of this gene is significantly different in humans and mice. It is assumed that

different splice variants evolve so that novel uses for the gene can be biologically tested without altering the functions the gene previously had[1172]. These new regulatory elements allow the gene to be under the control of different mechanisms in different parts of the body, naturally, therefore different *BDNF* splice variants are expressed in different parts of the brain likely under the control of different mechanisms[274]. Evolutionarily it makes sense for a higher organism to have stricter regulatory networks within the CNS regarding key proteins. The wider range of transcript variants demonstrates that this is true of BDNF in humans compared to mice. This begs the question then, do the differences in its regulation alter the clinical significance of findings associated with this gene? And assuming that they probably do have some significance, how does one deal with these issues when drawing conclusions about human illness based on animal work.

In addition to having significantly different genetics, mice exhibit different anatomical structures to humans. There are several key differences between the mouse and the human eye. The lack of the lamina cribrosa may be the source of interesting conundrum for rodent models of glaucomatous cell damage. The demonstration of deformed pores in the lamina cribrosa in glaucomatous human eyes, but not in healthy eyes[1173-1176], is proof that the lamina cribrosa is under stress in glaucoma. The stress on the lamina cribrosa likely provokes the response from the astrocytes that line the canals, responsible for producing the extra cellular matrix that makes up the lamina cribrosa. Indeed the astrocytes within the optic nerve head of glaucomatous eyes demonstrate broad signs of increased activity[1177] and begin producing elastin (which is absent in normal eyes)[1178]; this is likely responsible for the tissue remodelling seen in glaucoma. Although these changes have been well known for over 20 years, the interactions between optic nerve head astrocytes and RGCs are incompletely understood. Given that mice do not have a lamina cribrosa (despite functional astrocytes), it is clear that there will be different astrocyte-RCG interactions in mice, as compared to in humans, these interactions will be challenging, if not impossible, to evaluate in mouse models of glaucoma.

The hypothesis examined in this research does not explicitly implicate astrocytes although there is potential for astrocytes to be involved in the pathways assessed. At least in the brain, astrocytes have been seen to sequester BDNF prior to synaptic loss when axonal transport is interrupted[333]. If retinal astrocytes perform a similar role in the retina is not known, however if this was shown, then then applicability of the results to human illness would be difficult to prove given the differences in astrocytes between these species. Current

results that suggest the microbiome could play a role in astrocyte physiology include the finding from a PD model where a faecal matter transplant between mice led to shifts in the morphology of the astrocytes in the striatum[550]. Also, work has shown that the xenobiotic receptor AHR is activated in astrocytes by microbiome metabolites within the tryptophan pathway[1020]. That said, astrocytes were not noticeably altered in the substantial work by Erny *et al.* that identified the immature microglia phenotype in mice with an absent microbiome[540]. If the microbiome strongly impacts on the activity and function of astrocytes across the CNS, then it stands to reason that it is possible that the absence of a lamina cribrosa structure, which human astrocytes play an important role in maintaining, could indicate a potential issue in generalizability of these results.

Perhaps the most relevant question to ask regarding the external validity of results of ONC studies is if murine RGC's are representative of human RGC's. It is well known that different RGC types may have different propensities to cell death in glaucomatous damage[365, 1179, 1180], with the melanopsin expressing (intrinsically photosensitive) RGCs being most resilient[1179]. Langer *et al.* point out, as justification for their recent article investigating RGC diversity derived from stem cells, that "previous efforts have identified numerous RGC subtypes in animal models, but less attention has been paid to human RGCs"[1181]. A detailed review of the literature published in 2015 identified 30 RGC types in mice[1182], however the same depth of literature base simply doesn't exist for determining the extent of the diversity of human RGCs, although textbooks have stated that there are at least 18 morphological subtypes in humans[1183]. Nevertheless it is clear that the murine retina contains RGCs with expression patterns seemingly not found amongst human RGCs; In Langer's recent article, certain molecular markers that have characterized mouse RGCs were not found in human RGCs (derived experimentally from stem cells)[1181]. Similarly it appears that there are populations of RGCs unique to primates[1184]. Although this only begins to characterize the differences between the retinae of mice and men, it immediately suggests that RGC behaviour cross species may be different especially in response to diseases naturally present in one species but not the other. Similarly, the presence of the macula, with a very high density of RGCs[1183, 1185], responsible for high visual acuity in central vision, in humans, but not mice, suggests that the community structure of human RGCs is significantly different to that of a mouse, and although the cells involved are similar across species, caution is required before emphatically generalizing these results to humans.

The final aspect of validity that needs to be considered with regards to the generalizability of results is the differences in the response of mice and men to the same stimulus. One paper demonstrated that, based on the sequencing of whole blood samples, the genomic response to inflammatory stimulus in humans lead to reasonably predictable genomic response to a similar inflammatory response (i.e. the response to a burn, to trauma and to endotoxemia were each similar) however that the genomic response in mice to these stimulus did not correlate at all either to each other ($R^2 \leq 0.13$) or to human inflammation ($R^2 \leq 0.09$)[1186]. These poignant findings were sensationalized in the lay media and taken to suggest that that murine biology may be completely irrelevant to human illness. However, as Osterberg *et al.*[1187] and Shay *et al.*[1188] both pointed out, the findings by Seok *et al.* are taken from whole blood and, given that mice and humans have different abundances of cell types within their blood and the cellular response to inflammatory stimulus can be different, this is a confounding factor not addressed in the initial study. Furthermore Takao and Miyakawa[1189], and Shay *et al.*[1188] reported diametrically opposed conclusions in their reanalysis of the same data after excluding genes that were not significantly altered in either animal. The swift and strong reaction from other researchers, attempting to explain the results of Seok *et al.*, demonstrates just how important mice are to human disease researchers. Although one may be inclined to 'side' with those refuting Seok *et al.*, it is important to be aware of the strong internal bias that the 'sunk cost fallacy' provokes[1190]. Nevertheless, Seok *et al.* are not the only group to question the validity of mouse models for the investigation of human disease. The most common criticisms of animal models remain the failure of translation when assessing new therapeutic interventions[1191-1193]. It remains unclear why novel therapeutic agents seem to fail in human trials with such high frequency, and it is likely that multiple causes, ranging from internal validity issues, to interspecies biological differences, to publication bias, may account for the disparity in different circumstances. Even so, these translational issues broadly call into question the generalizability of animal models for human illness. Fundamentally, when it comes to glaucoma, this is evident in the lack of any neuroprotective intervention despite decades of research into neuroprotective strategies[15, 1194-1196].

It was shown that the microbiome's presence is beneficial for RGC survival in an ONC model, and this was confirmed in a cohort of CON mice. Similarly, treatment with a probiotic member of the normal microbiome (a strain of *L. Plantarum*) was able to partially correct the

RGC survival deficit in GF mice, and this required live microbes to have an effect. Although this work was undertaken with the hypothesis that baseline levels of BDNF protein in the retina may be varied at baseline, and therefore baseline levels of BDNF may be responsible for the findings, this was shown to be unlikely, at least at this simplistic level, in this research. In the research presented BDNF protein levels were shown to increase more readily in SPF and CON mice after an ONC by day 3, though, which may inform an interpretation of these results. To further add to this explanation, it was shown that BDNF injection into the eye obviated the difference between SPF and GF mice suggesting that this was able to saturate the BDNF related mechanisms such that the difference between SPF and GF mice in endogenous BDNF was irrelevant. Indeed, this is an area that requires more study beyond what is able to be presented in this thesis. This work, looking into the role of the microbiome on BDNF expression in the retina in GF mice, as compared to SPF mice, and how this is affected by optic nerve damage is ongoing and will likely form the majority of our laboratories future research in this area. It is clear that this aspect of the project is still within its preliminary stages and therefore only speculation can be offered at this stage. Nevertheless, the implications of the findings at present are exciting.

7.9 Germ Free Mice as a Model of Altered Microbiome

The GF mouse model is perhaps the cleanest model of altered microbiome currently available to microbiome researchers. Even so, it has received criticism for its generalizability; as such, it is worth investigating the potential issues with the GF model in more depth.

Of the models for microbiome disturbance, the GF model appears to be the most unadulterated model, requiring no pharmacological establishment, and appears to be mostly reversible with the relatively easy (and predictable) conventionalization process[883]. Indeed, GF models also form the basis of gnotobiotic models whereby the interactions between specific microbes and the host can be assessed, and specific communities can be formed to determine how microbiome components may work together in the health promotion of the holobiont. GF models are perhaps the most 'basic' of microbiome-host interaction studies, and for this reason they offer a moderately 'sensitive' approach to determining if the microbiome is involved in a biological process[883, 884]. More 'specific' research should follow. It is clear that the microbiome results presented in this thesis do not explain the specific findings

of the epidemiological findings, however they work with the epidemiological findings, in concert, pointing in favour of the central hypothesis of this thesis.

7.10 Optic Nerve Crush as a Model for Glaucomatous Cell Death

The discussion of the appropriateness of the ONC echo's the discussion of microbiome disturbance models. Indeed, each animal model of glaucomatous cell loss has limitations, and it is clear that the ONC is far from a 'perfect' model. Even so, it appears that the ONC offers a reliable model of RGC cell death and therefore it is sensitive to factors that alter the propensity for RGC's to die[907]. The ONC model is independent of IOP and can be used to directly assess how neuroprotective mechanisms may benefit the RGC loss seen in human illness.

Disease by its very nature is heterogenous, models of disease by their nature attempt to minimize heterogeneity to the greatest extent possible, so that generalizations about the effects on biological processes can be made and perhaps applied back to illness. It is no great surprise to say that biology is a messy science, a far cry from the purity of maths and physics. Standardization of disease conditions, however, allows for predictable and reliable 'normals' to be compared to interventional groups. These comparisons allow for the assessments of pathological mechanisms or potential therapeutic options in great detail. For this reason, reliable animal models with precise measurable variable are favourable for research in biology. Although these techniques can be technically challenging, the results of RGC death profile in these mice is highly reproducible, and therefore these represent a good model for assessing the potential for different agents/exposures to have even small effects on RGC survival[906].

The main drawback to these optic nerve injury models is that they initiate cell death in one swift insult, and although a progressive cell death occurs after the crush/transection, it is clear that there is a single initiating event. The single insult with a fairly coordinated and concurrent initiation of cell death in a large proportion of the RGCs is unlike human pathology where the dying cells undergo apoptosis at different time points over many years, with the vast majority of surrounding cells, at any particular timepoint, remaining healthy despite ongoing pathology[910]. Trading off homogeneity for biological equivalence is necessary in all animal research. ONC research cannot reveal if there is any interaction between the underlying factors being analysed and the long term cell-cell communication in a

glaucomatous retina, as the RGCs essentially die too rapidly for cell-cell communication to significantly interact, at least in any way that is relevant to human pathology.

Whilst the results from ONC models are interesting and prompt further work, the expense of a human clinical trial cannot be justified solely on ONC studies in rodents. However, these studies do inform the next steps of animal work. Indeed, in this work itself, the findings contribute evidence to the idea that the microbiome can protect the RGCs in glaucoma. The next steps therefore must address the mechanisms and clinical significance of this as a protective feature before human trials can be justified.

Chapter 8 – Conclusions

To briefly summarise, it was consistently demonstrated that IBS, an illness associated with abnormal microbiome and minimal specific host pathology, is associated with Glaucoma in three predominantly white adult populations. Where possible it was shown that IBS temporally precedes glaucoma development. It was also shown that recent tooth loss may increase the risk of developing glaucoma in the short term. Furthermore, it was shown that the microbiome's presence is associated with RGC survival in a murine ONC model of glaucoma. When the microbiome was restored the protection was restored, and when monocolonised with a live 'probiotic' strain of *Lactobacillus*, protection was conferred. Combined, these results suggest that the microbiome may play a role in RGC health, specifically in the context of the neurodegenerative processes involved in glaucoma.

Eye research, like all research, suffers from the relentless chase for a *p*-value below some arbitrary cutoff[1197]. This has led to research targeted towards what is pejoratively known as *p*-hacking. The research presented in this thesis, however, began as a hypothesis developed from a broad reading of the literature that progressed toward an eclectic suite of experiments that attempted to probe the hypothesis from different angles.

Epidemiology attempts to establish the relevance of a clinical question within the population at large. As has already been identified, the heterogeneity of human illness means epidemiology requires large samples and the difficulty of adequate variables means that compromises are often made, however the fundamental finding of the epidemiology work presented is the reliably of the findings, with multiple different variable definitions, IBS and perhaps dental illness, predisposes an individual to develop glaucoma. The epidemiology cannot explain the reason for this link or even if this link is strictly causal however as has been exhaustively articulated, the central effects of the microbiome are in the opinion of the team involved in performing this research, the best explanation for these findings, especially as the data pertain to IBS.

Animal research addresses the question from the opposite end of the spectrum and attempts to, through manipulation of only one or a few variables, determine variables that are relevant to the outcome measures. Indeed, the findings have demonstrated that an absence of microbiome leaves a mouse's retina more vulnerable to injury induced neurodegeneration. This effect can be rescued in a number of ways as mentioned previously

and the summary of these findings are that, at least in rodents, the microbiome has a neuroprotective effect on the optic nerve.

Combined, these findings have a clear theme in favour of the central *a priori* hypothesis. Although neither finding conclusively proves the central hypothesis, these findings lay significant groundwork for future work that can further investigate this question; the idea of microbiome based neuroprotective therapy should be exciting for neuroscientists and glaucoma doctors.

8.1 Implications for Neuroprotection Research

This work adds to an emerging body of work that is demonstrating a role for microbiome in the physiology of neurodegenerative illness. The extant literature relating glaucoma to the microbiome are minimal probably because glaucoma is relatively forgotten in neurodegenerative research due to esoteric nature of its neurology, mainly studied only by vision scientists.

As animal glaucoma models offer a decent model of injury induced neurodegeneration the findings add to a broader literature base and may be applicable to traumatic brain injury, chronic traumatic encephalopathy, and stroke. Previous work in ischaemic stroke showed that antibiotic induced microbiome suppression lead to decreased infarct volumes[542], which essentially suggests the opposite of the findings presented here.

Glaucoma has significant progress in novel therapeutic options in recent decades, although this work does not clearly indicate a therapeutic avenue for microbiome alteration in glaucoma, it does nudge toward a new avenue of investigation that could yield interesting therapeutic options surrounding a microbiome mediated neuroprotective pathway.

If one considers only the results linking IBS to the development of glaucoma, there are other questions which are raised with regards to potential neuroprotective avenues. IBS is well known to be linked to psychiatric comorbidity, the two have bidirectional relationship[812, 813] which may be due to the effects of the microbiome on CNS homeostasis. Indeed chronic illnesses are strongly associated with depression and anxiety[1198, 1199], in trend for which glaucoma is no exception[1200], however it is unclear if the psychological stress of chronic illness is responsible for the psychiatric outcomes or if underlying pathophysiology results in neurobiological dysfunction which manifests with psychiatric symptoms; it is likely that both play a role. Along this same line of thought, if IBS (or its associated dysbiosis) is responsible

for low level systemic inflammation as has been described by some studies[781-783], then perhaps this could be the underlying mechanism contributing to the neurobiology of both depression and anxiety in IBS patients and the predisposition to glaucoma. If this is the case anti-inflammatory therapeutics may eventually play a role in the treatment of glaucoma.

One can conclude that if IBS precipitates glaucoma, then either microbiome disturbances or low-grade inflammation contribute to glaucoma, and therefore if ongoing microbiome research is fruitless that this inflammatory state (which incidentally may also likely be caused by dysbiosis) should be investigated further. Nevertheless, since the animal research presented demonstrates a microbiome mediated neuroprotective effect, and the evidence for systemic inflammation as a cause of glaucoma is limited, in all likelihood, the effect of IBS on glaucoma is likely due to microbiome effects.

8.2 Future Work Required

The epidemiological relationships presented in this thesis were theorised based on the underlying physiological interactions between the microbiome and the CNS. Whilst the animal model research lends credibility to these findings, there remains a gap in the research regarding the specifics of the microbiome in people with glaucoma. Indeed, these studies are complex and data-intensive, however it will be important to confirm if microbiome abnormalities are a feature of glaucoma. Similarly, as noted, IBS subtypes may each have microbiome abnormalities, and so future epidemiological research into the IBS-glaucoma relationship should attempt to identify the subtypes associated with glaucoma.

The animal model research presented was unable to determine a mechanism linking the microbiome to neuroprotection; however, this is an ongoing issue with microbiome-host interactions. Indeed, it seems as if some unidentified circulating agent, which can pass through the BBB, or at least signal through the BBB, is having broad effects on the CNS, implied in this research through the microbiome's effects on RGC loss in ONC models. Identifying the mechanism(s) that the microbiome enact is highly important for development of targeted drug therapies. However, perhaps as understanding of dysbiosis grows more general therapies surrounding the boosting of microbiome health will have effects in glaucoma, amongst the other illnesses that seem to result from poor gut health. Indeed, this does require a certain level of interest in developing understanding around measurement and categorisation of dysbiosis which has been difficult to this point and remains largely an

ignored problem as published p values are low for the research that is being produced with the methods that are currently available.

Some have demonstrated some effects mediated through circulating nutrients. The microbiome is highly metabolically active and is responsible for the metabolism of various nutrients such as starches for which the host has no metabolic capacity. These metabolic processes result in the production of metabolites which may have nutritional value, and interestingly some of these may play a ligand role in some receptors in the host. The most notable of these pathways is the SCFA production which occurs when the microbiome breaks down starches[1201]. These products, of which butyrate, propionate and acetate are notable examples, have various effects on the human physiology. Notably, it has been shown that microglia maturation requires SCFA's, normally produced by the microbiome, to progress normally[540]. I hypothesized that since SCFAs were involved in microglial maturation, able to virtually obviate the GF SPF differences seen in that study, and that since they have been shown to positively regulate BDNF in the central nervous system[381, 1202], it was possible that they could play a role in mediating the microbiome effects. However, preliminary research, using the same SCFA protocol published in that paper[540], found no benefit in RGC survival after ONC, suggesting at least preliminarily that these chemicals are not relevant to the neuroprotective microbiome-retina interactions noted (due to the incompleteness of this work, data are not presented in this thesis).

Although SCFA's are some of the most numerous of the microbiome produced metabolites many other classes of metabolites are released from the microbiome which could possibly affect CNS. In the pursuit of this project I investigated this further with specific reference to the AHR, leading to the review article reproduced in Chapter 2. The AHR responds to a number of microbiome mediated compounds including those of the indole[1203] and kynurenine[1204] metabolite families, suggesting AHR's role in sensing microbiome signalling. In preliminary work, unpublished in this thesis, it was found that the AHR itself is not expressed, to any significant degree, in adult murine RGCs. Other research has similarly found that its expression in the retina is at the pigmented epithelium and not in the ganglion cells[1205], leading to its examination in other retinal illnesses[1019, 1205]. Nevertheless, its role in the neuroimmune system may be relevant to glaucomatous pathology and could be investigated in future studies. It remains to be seen if the microbiome effect seen in GF and

SPF wild type mice, described in Chapter 6, would also be seen in *Ahr* knock-out mice. This is certainly an area for future investigation.

Others have demonstrated that the ENS which communicates with the CNS via the vagus nerve and the sympathetic column may have some responsibility in transmitting microbiome signals to the brain[517, 1206]. Regarding this issue, it is interesting that seemingly conflicting experiments have been published[516]. It seems likely that there is some microbiome-CNS mediated communication that occurs through the vagus nerve, but it is also clear that other effects are mediated by vagus independent mechanisms[516]. It is currently not determined if there is redundancy in the overlap between vagus mediated mechanisms and ligand/biochemical mediated mechanisms. The potential for vagus signalling to be involved in microbiome-retinal interactions seems unlikely given the remote connection from the nuclei of the vagus to the optic nerve. Nevertheless, it seems likely that for other neurodegenerative illnesses, namely Parkinson's disease, the vagus is a direct avenue through which pathology gains access to the brain.

Indeed, to delve into pathway identification will require a great deal of investment in unbiased techniques. Identification of implicated pathways in extremely complex systems such as the holobiont, without a clear candidate physiological mechanism, requires bioinformatic analyses of the total breadth of measurable pathways to reveal those which are most correlated to the outcomes of this ongoing work. Following the identification of those pathways most statistically relevant to the microbiome host interaction of interest the biological plausibility of these will then need to be assessed through targeted research models. This complex, time and resource consuming avenue of research will be necessary in the coming era of holobiont research.

With regards to glaucoma the next steps regarding the identification of microbiome interaction with disease prevalence, incidence and severity will require human epidemiological studies. Although the downfalls of contemporary microbiome research have been clearly discussed in Section 1 of this thesis, there is certainly utility in applying these techniques to the human glaucoma question. As large cohorts are being formed for the understanding of human disease, especially those with longitudinal follow-up, there is significant potential for the differences in microbiome composition in people with and without glaucoma to be assessed. As better techniques are developed to understand the health of the microbiome the results will be better able to show this. The use of the

pathomarker should ideally be superseded by techniques that better identify dysbiosis. Perhaps the most interesting work will be done in the area surrounding the interactions between microbiome activity and host genetics. After all, as genetic research moves from identification of mutations in specific genes of indeterminate action toward the identification of gene-gene interactions, for which biological inferences are more obvious, in illnesses[1207], similar research will begin in microbe-microbe interactions as well as microbe-gene interactions, and even more sophisticated models as computational biology improves.

It is clear that the findings presented are a meagre appetizer in comparison to the wealth of potential information that they hint towards. Nevertheless, the identification that a distant and supposedly contained system such as the microbiome could be affecting the neuroprotective mechanisms in the retina is exciting for its implications in human understanding of both the holobiont and glaucoma pathology itself.

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Appendices

Appendix 1: Permission to Re-Print Article Published in the Journal of Molecular Medicine

Title: Host-microbiome interactions: the aryl hydrocarbon receptor and the central nervous system

Author: Hae Ung Lee, Zachary E McPherson, Bryan Tan *et al*

Publication: Journal of Molecular Medicine

Publisher: Springer Nature

Date: Jan 1, 2016

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Appendix 2: Survey Sent to ANZRAG Cohort

Hunter Community Study/Australian and New Zealand Registry of Advanced Glaucoma
Gastrointestinal Health Survey

Questions 1-3 ask you about any pain or discomfort in your abdomen, stomach or tummy or bowel problems that you may have had in the past 12 months. Please do not count cramps or pain with menstrual periods and do not count pain in your chest.

1 **In the last 3 months, how often did you have discomfort or pain anywhere in your abdomen, stomach or tummy?**

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

2 **Did you only have pain in your abdomen, stomach or tummy (not discomfort or a mixture of pain and discomfort)?**

- ○ No
- ○ Yes

3 **During periods when you had ANY pain or discomfort in your abdomen, stomach or tummy, how often would you say that:**

Use the following options to help you answer these questions.

Not at all
Sometimes: (less than one quarter (25%) of the time)
Often: *(more than one quarter (25%) of the time)*
Very Often: (more than half (50%) of the time)
Almost Always: (more than three quarters (75%) of the time)

		Not at all	Sometimes	Often	Very often	Almost always
A	The pain or discomfort was made **better or stopped** by having a bowel movement?	○	○	○	○	○
B	You had **more** bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○
C	You had **less** bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○
d	You had **looser** bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○
e	You had **harder** bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○

4 **In the last 12 months, have you had any pain, discomfort or burning in your abdomen, stomach or tummy that was usually in a single small area that you could point to with one or two fingers (above your belly button)?**

- ○ No
- ○ Yes

5 **In the last 3 months, how often have you had any pain, discomfort or burning in your abdomen, stomach or tummy that was usually in a single small area that you could point to with one or two fingers (above your belly button)?**

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

5 **In the last 12 months, have you ever felt an uncomfortable fullness soon after starting to eat that you could not finish a normal meal?**

- ○ No
- ○ Yes

6 **In the last 3 months, how often did you feel an uncomfortable fullness soon after starting to eat that you could not finish a normal meal?**

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

7 **In the last 12 months, have you been unable to finish a normal meal?**

- ○ No
- ○ Yes

8 **In the last 3 months, have you been unable to finish a normal meal?**

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

9 **Which <u>one</u> of the following <u>best</u> describes your usual way of eating?**

- ○ No special way of eating
- ○ Vegetarian
- ○ Weight Reduction Diet
- ○ Diabetic Diet
- ○ Fat Modified diet to lower blood fat (Cholesterol)
- ○ Other (Please Specify): _____

10 **In the last 12 months, How often have you consumed 250ml of milk (Skim Milk, Low fat milk or Whole milk)**

- ○ Never
- ○ Less than 1 per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2-3 per day
- ○ 4+ per day

11 **Have you used eye drops for your glaucoma within the last 3 months?**

- ○ Yes (please specify below)
- ○ No

12 **Please list the names of the eyedrops, from the bottle, that you have used within the last 3 months:**

_____

_____

_____

_____

Appendix 3: Survey Sent to HCS Cohort

Questions 102 – 115 ask you about any pain or discomfort in your abdomen, stomach or tummy or bowel problems that you may have had in the past 12 months. Please do not count cramps or pain with menstrual periods and do not count pain in your chest.

102. In the last 12 months, have you ever had any pain or discomfort in your abdomen, stomach or tummy?

- ○ No → **Go to Q104**
- ○ Yes

103. In the last 3 months, how often did you have discomfort or pain anywhere in your abdomen, stomach or tummy?

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

104. In the last 12 months, have you had any pain, discomfort or burning in your abdomen, stomach or tummy that was usually in a single small area that you could point to with one or two fingers (above your belly button)?

- ○ No → **Go to Q108**
- ○ Yes

105. In the last 3 months, how often have you had any pain, discomfort or burning in your abdomen, stomach or tummy that was usually in a single small area that you could point to with one or two fingers (above your belly button)?

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

106. Did you only have pain in your abdomen, stomach or tummy (not discomfort or a mixture of pain and discomfort)?

- ○ No
- ○ Yes

107. During periods when you had ANY pain or discomfort in your abdomen, stomach or tummy, how often would you say that:

Use the following options to help you answer these questions.
- Not at all
- Sometimes: (less than one quarter (25%) of the time)
- Often: (more than one quarter (25%) of the time)
- Very Often: (more than half (50%) of the time)
- Almost Always: (more than three quarters (75%) of the time)

	Not at all	Some-times	Often	Very often	Almost always
a. The pain or discomfort was made better or stopped by having a bowel movement?	○	○	○	○	○
b. You had more bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○
c. You had less bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○
d. You had looser bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○
e. You had harder bowel motions (stools) than usual when the pain or discomfort began?	○	○	○	○	○

108. In the last 12 months, have you ever felt an uncomfortable fullness soon after starting to eat that you could not finish a normal meal?

- ○ No → **Go to Q110**
- ○ Yes

109. In the last 3 months, how often did you feel an uncomfortable fullness soon after starting to eat that you could not finish a normal meal?

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

110. In the last 12 months, have you been unable to finish a normal meal?

- ○ No → **Go to Q112**
- ○ Yes

111. In the last 3 months, how often were you unable to finish a normal meal?

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

112. In the last 12 months, have you ever leaked or passed bowel motion (stools) at unwanted times?

- ○ No → **Go to Q114**
- ○ Yes

113. In the last 3 months, how often did you leak or pass bowel motion (stools) at unwanted times?

- ○ Never
- ○ Less than one day a month
- ○ One day a month
- ○ Two to three days a month
- ○ One day a week
- ○ More than one day a week
- ○ Every day

114. In the last 3 months, how often did you have any of the following problems with your bowels?

Use the following options to help you answer these questions.
- Not at all
- Sometimes: (less than one quarter (25%) of the time)
- Often: (more than one quarter (25%) of the time)
- Very Often: (more than half (50%) of the time)
- Almost Always: (more than three quarters (75%) of the time)

In the last three months, how often have...	Not at all	Some-times	Often	Very often	Almost always
a. You had more than three bowel motions each day?	○	○	○	○	○
b. You had less than three (0-2) bowel motions each week?	○	○	○	○	○
c. Your stools were very lumpy or hard?	○	○	○	○	○
d. Your stools were very loose or watery?	○	○	○	○	○
e. You needed to strain a lot to have a bowel motion?	○	○	○	○	○
f. You experienced an urgent need to have a bowel motion that made you rush to a toilet?	○	○	○	○	○
g. After finishing a bowel movement you felt that there was still bowel motion (stools) that needed to be passed?	○	○	○	○	○

Q114 continues on page 22

114. *continued...*

In the last three months, how often have...

	Not at all	Some-times	Often	Very often	Almost always
h. You had a sensation that the stool could not be passed (e.g. blocked) when having a bowel motion?	○	○	○	○	○
i. You had difficulty relaxing or letting go to allow stool to come out during a bowel motion?	○	○	○	○	○
j. You needed to press your finger in or around the anus (back passage) or vagina (front passage) to help the bowel motion to come out?	○	○	○	○	○

115. Did you have any of the above bowel problems in Q114 (a-j) in the last 12 months?
- ○ No
- ○ Yes

Questions 116 – 126 ask about your use of alcohol.

116. How often do you have a drink containing alcohol?
If 'Never' → **Go to Q127**

Never	Monthly or less	2–4 times a week	2–3 times a week	4 or more times a week
○	○	○	○	○

117. How many drinks containing alcohol do you have on a typical day when you are drinking?

1–2	3–4	5–6	7–9	10 or more
○	○	○	○	○

118. How often do you have six or more drinks on one occasion?
- ○ Never
- ○ Less than monthly
- ○ Monthly
- ○ Weekly
- ○ Daily or almost daily

119. How often during the last year have you found that were not able to stop drinking once you had started?
- ○ Never
- ○ Less than monthly
- ○ Monthly
- ○ Weekly
- ○ Daily or almost daily

120. How often during the last year have you failed to do what was normally expected of you because of drinking?
- ○ Never
- ○ Less than monthly
- ○ Monthly
- ○ Weekly
- ○ Daily or almost daily

121. How often during the last year have you found it difficult to get the thought of alcohol out of your mind?
- ○ Never
- ○ Less than monthly
- ○ Monthly
- ○ Weekly
- ○ Daily or almost daily

122. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?
- ○ Never
- ○ Less than monthly
- ○ Monthly
- ○ Weekly
- ○ Daily or almost daily

Appendix 4: Supplementary Methods for Chapter 3

Identification of IBS

The modified ROME III IBS questionnaire has been used previously with the following considerations[1208]. IBS was defined by abdominal pain or discomfort at least 2-3 days per month for the past 3 months, with at least two associated symptoms:

- Improvement of pain with defecation
- Onset of pain associated with a change in the constancy of bowel motions
- Onset of pain associated with Alterations in bowel motion frequency

The present questionnaire as well as the standard Rome III criteria[1209] use a 5 point Likert scale. However, the present study also offered a guide that provided a numerical definition of each. The scale provided was as follows:

Score	Rome III	HCS/ANZRAG
0	Never or Rarely	Not at all
1	Sometimes	Sometimes (less than one quarter (25%) of the time)
2	Often	Often (more than one quarter (25%) of the time)
3	Most of the time	Very Often (more than half (50%) of the time)
4	Always	Almost Always (more than three quarters (75%) of the time)

Although the above numerical guides were only provided at the top of the questionnaire, it is conceivable that these scales could be interpreted slightly differently by participants. For this reason, although the ROME-III criteria set the limit at "Sometimes"[1209], which for the present study is also used for the 'conventional' IBS definition, a 'stringent' definition was also created with "Often" as the cut-off. The stringent definition acted as a sensitivity analysis for the analyses.

Appendix 5: Supplementary Material for Manuscript Presented in Chapter 4

Supplementary Methods

Identification of IBS in UKBC

At age 42, participants were asked "*Have you ever had or been told you had Irritable bowel syndrome or IBS?*". A participant who answered "Yes" to this question was considered an IBS case at or before the age of 42.

At age 50, participants were asked "*Are you currently suffering from any of the health problems listed on this card?*"; if they indicated that they had '*Problems with stomach, bowels or gall bladder',* they were asked the question "*You say you have stomach, bowel or gall bladder problems. Looking at this card, can you tell me which of these conditions you have?*". A response of '*Irritable bowel syndrome or IBS*' identified a case of IBS at age 50. For both questions, multiple answers, indicating that a participant suffered from multiple conditions, were accepted.

Identification of Glaucoma in UKBC

At age 42, participants were asked "*Since [the previous survey that the participant participated in], have you had or developed any problem with your eyesight or any abnormal eye condition?".* If they responded *"Yes, sight or eye problem in both eyes"* or *"Yes, sight or eye problem in one eye only",* participants were asked *"What is or was wrong with your vision or eyes?".* An answer of "*Glaucoma - vision problems resulting from increased pressure in the eye*" indicated a case of glaucoma at age 42. At age 50, participants were asked *"Are you currently suffering from any of the health problems listed on this card?"* If they responded *"Problems with eyesight including wearing glasses or contact lenses",* they were asked "*You said you have problems with eyesight. What is wrong with your vision?".* An answer of *"Glaucoma (You have a 'raised' pressure in the eyes)"* indicated a case of glaucoma at age 50.

Supplemental Table 4.1. ICD-8, ICD-10, procedure Codes, and ATC medication codes used to identify patients, ascertain exposures, and define covariables in the DNPR cohort.

Variable	Codes
Irritable Bowel Syndrome	ICD-8: 564.19 ICD-10: K58.0, K58.9
Cholelithiasis (negative comparison cohort)	ICD-8: 574, 575 ICD-10: K80
Glaucoma (Hospital diagnosis)	ICD-8: 37510-37519 ICD-10: H40.1
Glaucoma (Surgical intervention)	Procedure codes: KCHD, KCHF05, KCHF10, KCHF15, KCHF20, KCHF30, KCHF99
Glaucoma (Redeemed prescription)	ATC: S01E, except S01EC01
Diabetes mellitus	ICD-8: 249, 250 ICD-10: E10, E11, E12, E13, E14 ATC: A10A, A10B
Sleep apnoea	ICD-10: G47.3
Steroid usage (Redeemed prescription)	ATC: H02
Chronic Obstructive Pulmonary Disease	ICD-8: 490-493; 515-518 ICD-10: J40-J47; J60-J67; J68.4; J70.1; J70.3; J84.1; J92.0; J96.1; J98.2; J98.3

Supplemental Table 4.2. COPD prevalence in IBS patients identified from the DNPR and their matched controls.

	IBS cohort (n=62,541)	Matched general population cohort (n=625,410)	Cholelithiasis cohort (hospital comparison cohort) (n=62,540)
COPD	4,549 (7.3%)	25,144 (4.0%)	3,781 (6.1%)

Supplemental Table 4.3: Results for IBS patients identified from the Danish National Registry of Patients compared to a general population comparison cohort, further adjusted by COPD

Glaucoma Definition	Cumulative Incidence Risk		Unadjusted hazard ratio	Adjusted hazard ratio*
	General population cohort	IBS patients		
Physician diagnosis	0.47 (0.43-0.50)	0.72(0.53-0.95)	**1.36 (1.16-1.59)**	**1.35 (1.15-1.58)**
Physician diagnosis (lagged)	0.45 (0.42 - 0.49)	0.70 (0.51 - 0.93)	**1.32 (1.11–1.56)**	**1.30 (1.10–1.54)**
Glaucoma surgery	0.24 (0.20-0.28)	0.28 (0.20-0.38)	**1.37 (1.06-177)**	**1.35 (1.04-1.74)**
Glaucoma surgery (lagged)	0.24 (0.20 - 0.28)	0.27 (0.19 - 0.37)	**1.33 (1.02–1.73)**	**1.32 (1.01–1.71)**
Glaucoma medication initiation	1.01 (0.94-1.09)	1.11 (0.94-1.30)	**1.21 (1.03-1.41)**	**1.19 (1.02-1.39)**
Glaucoma medication initiation (lagged)	0.94 (0.87 - 1.02)	1.04 (0.87 - 1.23)	**1.23 (1.04–1.45)**	**1.21 (1.03-1.44)**

Data with 95% confidence intervals are presented for both the complete analysis and for the 1-year lagged sensitivity analysis. *Adjusted for diabetes mellitus, sleep apnea, and COPD.

Supplemental Table 4.4. Results from the Danish National Registry of Patients: Risk of glaucoma in persons with IBS compared to those with cholelithiasis, further adjusted by COPD

Glaucoma definition	Cumulative Incidence risk		Unadjusted hazard ratio	Adjusted hazard ratio
	Cholelithiasis cohort	IBS cohort		
Physician diagnosis	0.53 (0.43 - 0.66)	0.72 (0.53- 0.95)	1.24 (0.97– 1.57)	1.24 (0.97– 1.58)
Glaucoma surgery	0.18 (0.12 - 0.26)	0.28 (0.20- 0.38)	**1.59 (1.06– 2.41)**	**1.69 (1.10– 2.60)**
Glaucoma medication initiation	1.11 (0.86 - 1.40)	1.11 (0.94- 1.30)	**1.26 (1.00– 1.59)**	**1.29 (1.01– 1.64)**

Data are presented with 95% confidence intervals.

Appendix 6: Permission to Re-Print Article Published in Ophthalmology

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Appendix 7: Achieved Power Calculations for Epidemiological Research

Below are the achieved power calculations for each of the studies presented in this thesis. All calculations were performed with G*Power 3.1[1210] (Universität Düsseldorf, Germany)

Power Calculation – ANZRAG/HCS study

Assumptions:

Exact test of Inequality of proportions in independent groups

Sample size group1: 1021 (ANZRAG cohort)

Sample size group 2: 2251 (HCS cohort)

Proportion positive (for IBS) in group 2: 11.3%

accepted α error: 0.05

Achieved power (1-β) for hypothesis (OR: 1.5): 0.958

Achieved power (1-β) for primary result (OR: 1.93): 0.999

Power Calculation – UKBC study

Assumptions:

Exact test of Inequality of proportions in independent groups

Sample size group1: 778 (people with IBS at/or before age 42)

Sample size group 2: 8313 (people without IBS)

Proportion positive (for incident glaucoma by age 50) in group 2: 0.5%

accepted α error: 0.05

Achieved power (1-β) for hypothesis (OR: 1.5): 0.182

Achieved power (1-β) for primary result (OR: 1.96): 0.406

Power Calculation – DNPR

Assumptions:

Exact test of Inequality of proportions in independent groups

Sample size group1: 62,541 (people with IBS)

Sample size group 2: 625,410 (population controls)

Proportion positive (for incident glaucoma diagnosis by physician) in group 2: 0.02%

accepted α error: 0.05

Achieved power (1-β) for hypothesis (OR: 1.5): 0.997

Achieved power (1-β) for primary result (OR: 1.31): 0.877

Power Calculation – HPFS study

Assumptions:

Exact test of Inequality of proportions in independent groups

Sample size group1: 55,215 (follow up years for people with periodontal disease)

Sample size group 2: 298,154 (follow up years for healthy controls)

Proportion positive (for incident glaucoma diagnosis by physician) in group 2: 0.08%

accepted α error: 0.05

Achieved power (1-β) for hypothesis (OR: 1.5): 0.802

Exact test of Inequality of proportions in independent groups

Sample size group1: 34,863 (follow up years for people with recent tooth loss)

Sample size group 2: 281,777 (follow up years for healthy controls)

Proportion positive (for incident glaucoma diagnosis by physician) in group 2: 0.09%

accepted α error: 0.05

Achieved power (1-β) for hypothesis (OR: 1.5): 0.699

Achieved power (1-β) for primary result (OR: 1.45): 0.622