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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Zachary E. McPherson

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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Zachary E. McPherson

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

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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.