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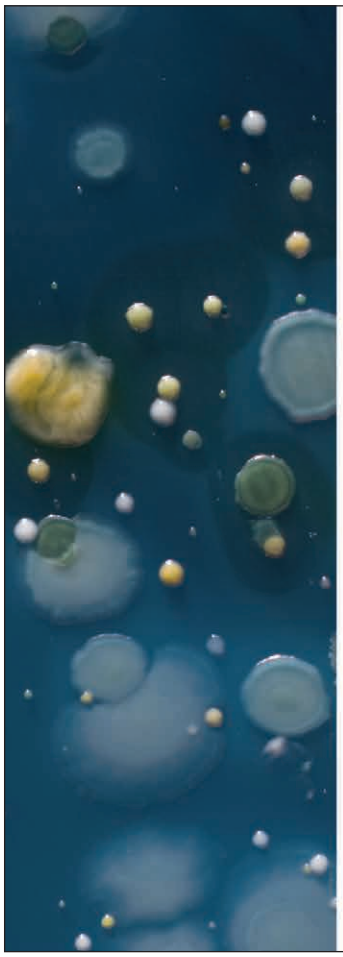
Human and microbial interactions that influence health outcomes



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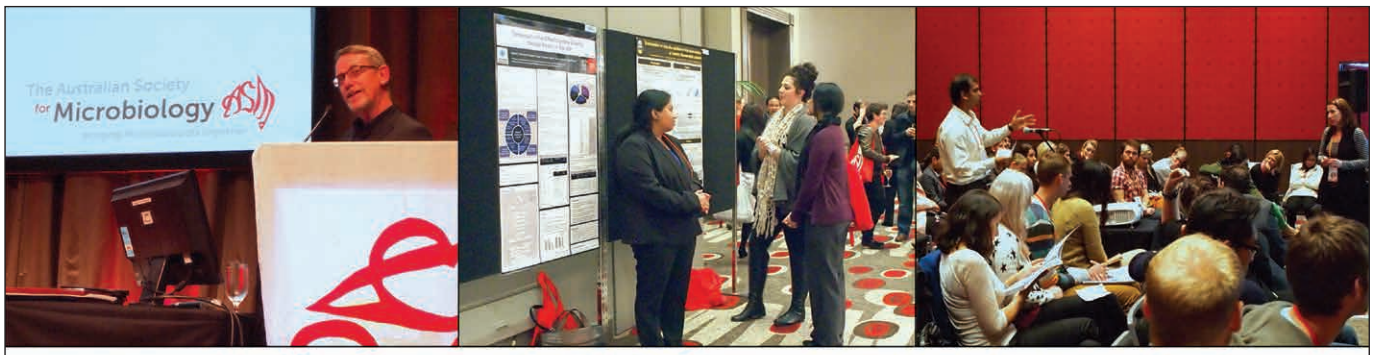

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Contents

Cover image: RAW264.7 murine macrophages infected with B. pseudomallei *for 12 hours.* B. pseudomallei*-induced host cell membrane fusion results in multi-nucleated giant cells. Host cell actin is polymerised by* B. pseudomallei *to form actin tails to propel bacteria into neighbouring cells. Macrophage nuclei are stained blue,* B. pseudomallei *are stained green and actin is stained red. The image was produced by Ms Nicole Bzdyl, UWA PhD candidate of Dr Tim Inglis, Dr Mitali Sarkar-Tyson and Dr Charlene Kahler.*

Dena Lyras
President of ASM

Last year was a very difficult year for many of you, and the anxiety, lockdowns and travel restrictions that we faced left us feeling unsure and grieving for a world we lost. We were all disappointed by the unprecedented postponement of our meeting in 2020. Until a few days ago, we thought that we would be able to hold our 2021 meeting in a face-to-face format – it is crushing that we now cannot do that. I am sad and upset that we will not be able to come together this year in the way we had anticipated.

However, a positive outcome from the restrictions of the last year was the development of excellent online conferencing tools. Our meeting this year was to be our first hybrid meeting, with the expectation that it would be delivered both face-to-face and online. Our contingency planning involved the scenario which has arisen and we have therefore switched the conference to a full online format, which also allows regional members and those not able to travel to attend the meeting. This conference format is new and gives us the opportunity to learn and socialise in different ways. I anticipate that some things will work and others will need refinement – we are all learning as we go!

I am proud of the hardworking and dedicated Local Organising Committee team which has spent the last three years putting together a wonderful and diverse scientific and social program for our enjoyment. I would like to congratulate and thank them for their tireless work during a very difficult few years, and sympathise with them over our thwarted plans.

I would also like to thank our Scientific Executive Committee, State Branch Committees, and EduCon Committee for their work towards developing and delivering online events; their efforts have gone a long way towards bringing our community together during a very difficult time and will bring lasting change to the Society.

I also encourage you to attend the World Microbe Forum, a worldwide online meeting being held on 20–24 June 2021 (https://www.worldmicrobeforum.org/). The theme of this meeting is Microbial Science Knows No Borders and it is a collaborative effort between nine Microbiological Societies from around the world, including ours, and discounted registration is available to our members. I thank A/Professor Kate Seib for the prominent role she has played in leading the organisation of the Australian arm of this meeting. The program is diverse and has something for everyone – do take a moment to have a look at the program on the website.

Finally, I extend my heartfelt congratulations to the recipients of our ASM awards for 2021. Please take a moment to view the gallery featuring our award recipients on our website. You can read a short biography of each recipient by clicking on their photo and we provide links to their LinkedIn profiles so you can easily send them your congratulations. Our winners are as follows – congratulations!

David White Excellence in Teaching Award – Meredith Hughes
Frank Fenner Award – John Atack and Nichollas Scott
Jim Pittard Early Career Award – Danielle Ingle and Jennifer Wood
Distinguished Service Award – Melissa Brown, Deirdre Mikkelsen and Jacqueline Schooneveldt
Teachers Travel Award – Thiru Vanniasinkam
Nancy Millis Student Awards – Laurine Kaul, Cheryl Sia, Elizabeth Peterson, Sarah Cahill, and Korakrit Imwattana

Don't forget to go to our new ASM Community portal, which allows members to connect with one another, to join special interest groups, have discussions with members who have similar interests, and to keep up to date on all ASM matters. To join the ASM community, go to https://community.theasm.org.au/ and click on the icon at the top RHS of the screen to set up your profile. Select 'Communities' from the banner menu to join a Special Interest Group community. Our other platforms, including our website www.theasm.org.au, ASM on Twitter, @AUSSOCMIC, or on Facebook, are also very active. We encourage and welcome your engagement using any option that suits you.

MICROBIOLOGY AUSTRALIA, 2021, **42**, 59
https://doi.org/10.1071/MA21018

Guest Editorial

Human and microbial interactions that influence health outcomes

Charlene Kahler and David Smith

Welcome to this edition of *Microbiology Australia* on human and microbial interactions that influence health outcomes.

As we understand the microbial world in all its diversity, we are now aware more than ever, that there is an amazing spectrum of interactions between the microbial community and the human body, which influences chronic health conditions. In the preceding decade, many large-scale studies have established that there is homeostasis between the host and microbiome which results in a tendency to resist change in order to maintain a stable, relatively constant internal environment. This relationship is maintained by a complex web of interrelationships in the microbiome itself, which secreted metabolic products that are detected by the host as a means of sampling the environment. When the microbiome is perturbed, a dysbiotic relationship between the two systems results in deleterious effects on human health such as the development of allergies, chronic inflammatory syndromes and even behavioural changes.

In this issue, we have short articles describing what we currently understand about the microbiome of the human gut and the cervicovaginal compartments. Dr Erin Shanahan explains how the gut microbial ecosystem is primarily altered by nutrient availability and that diet therefore represents an important asset in therapeutically altering the gut microbiome. Dr Willa Huston describes the role of the cervicovaginal microbiome, which is extremely important for maintenance of an acidic environment, preventing pathogenic colonisation, and modulates inflammation by cross-kingdom signalling. Thus, the composition of cervicovaginal microbiome plays an important role in health outcomes for women particularly in relation to vaginal infection, pregnancy, and fertility. Dr Jeff Keelan extends this theme by examining the potential of microbial profiling as a means of identifying women at risk of early pre-term birth which will assist in early interventions to improve neonatal survival.

A further two articles explore the concepts of using our knowledge of the human microbiome to inform novel intervention strategies for disease by using closely related species as a means of preventing unhealthy microbiome communities from developing. Dr Lea-Ann Kirkham describes the multiple mechanisms that are now being deployed to intervene in otitis media, which is caused by a polymicrobial biofilm in the inner ear. In my article on the *Neisseria* genus, I provide an update on recent expansion of this genus and provide a commentary on the importance of the commensal species in this group as a potential source of probiotics to inhibit meningococcal carriage and gonorrhoea.

As our understanding of the human microbiome and its role in developing tolergenic immune responses has matured, it has also become clear that historical infections affect the intensity of disease outcomes from certain infections. Dr Allison Imrie describes this feature in relation to outcomes for dengue infections. Cross reactive T-cell responses may drive either resolution of the infection or drive a life-threatening haemorrhagic response. Similar themes may emerge as we understand SARS-CoV-2 responses and why some people have mild symptoms while in others it is life threatening. Professor Ian Macreadie explores the role of dietary cholesterol and the use of statins to moderate the outcomes from a variety of respiratory viral infections including influenza and SARS-CoV-2.

Last, we have two articles providing an update on two difficult to treat multi-drug resistant pathogens, *Helicobacter pylori* by Professor Barry Marshall and *Burkholderia pseudomallei* by Dr Tim Inglis.

Biographies

The biography for **Associate Professor Charlene Kahler** is on page 83.

Clinical Professor David Smith, BMedSc, MBBS, FRCPA, FACTM, FASM, FFSc(RCPA), is a graduate in Medicine from the University of Western Australia and trained in Medical Microbiology in Perth. He is a Medical Virologist at PathWest Laboratory Medicine WA at the QE2 Medical Centre in Perth, Australia, where he is a Director of the Arbovirus Research Laboratory. He is also a Clinical Professor in the Faculty of Health and Medical Sciences at the University of Western Australia. Professor Smith serves on a number of state, national and international committees and advisory groups, and is currently Chair of the National Arbovirus and Malaria Advisory Committee. He has a particular interest in public health issues, including mosquito-borne viruses, influenza and other respiratory viruses, and emerging infections.

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https://doi.org/10.1071/MA21026

Munching microbes: diet–microbiome interactions shape gut health and cancer outcomes

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Abstract. The gut microbiome describes the complex community of microorganisms that populate the gastrointestinal tract. Gut microbes in the large bowel utilise both dietary-derived nutrients, such as host-indigestible carbohydrates (fibre) and excess protein, host-derived nutrients (intestinal mucin), and also interact with the by-products of digestion such as bile acids. They transform these compounds into a series of metabolites that can profoundly shape host physiology both locally and systemically. These metabolites can fundamentally alter host outcomes, promoting either gut health, or sub-optimal conditions in the gut that contribute to poor health, including increased risk of cancer. The microbiome of an individual has also been shown to impact response to cancer treatment strategies, including both treatment efficacy and side-effects in the gut and more systemically. This makes the microbiome a powerful potential tool for therapeutic purposes, once we overcome the challenges associated with individual variation in microbial community composition. As the gut microbial ecosystem is primarily altered by nutrient availability, diet therefore represents an important asset in therapeutically altering the gut microbiome.

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Consumption of a sub-optimal, Western-style diet (WD) – containing proteins from processed meats, saturated fats, refined grains and sugars, while lacking plant-derived, fibre-containing components[1] – has been identified as a key driver of various disadvantageous health states such as colorectal cancer (CRC)[2–4], obesity[5], Crohn's disease[6], and irritable bowel syndrome (IBS)[7]. Researchers have been seeking to understand the mechanisms behind these significant associations, including the impact of diet on the microbiome and the relationship between gut microbes and their host, particularly the epithelial barrier.

When examining the relationship between diet and the gut microbiome it is important to consider the nature of the gut environment and nutrient availability from the perspective of microbes. After digestion and absorption in the small intestine (Figure 1), the nutrients available to microbes in the large bowel are those unable to be digested by host enzymes, those surplus to requirements, or derived from host cells. Different microorganisms will have varying preferences and capabilities for consumption of dietary or host-derived carbohydrates and proteins[8,9]. Interactions between nutrients and the ratios of macronutrients available are also important in favouring the growth of microbes with particular nutritional strategies[10]. Therefore, in the context of the large bowel, which is the primary site of microbial fermentation in the gut, overall dietary intake will shape microbial community composition.

The intestinal epithelium and mucosal layer is a key site of interaction between the host, dietary nutrients and gut microbes[11]. It is a physical and immunological barrier and plays a fundamental role in the maintenance of host health and disease prevention. The layer of epithelial cells separates the luminal contents of the gut, including microbes, from the underlying tissue[12]. The epithelial layer itself is protected by a mucin layer, which prevents direct contact with microbial cells (Figure 2). A number of microbes are able to cleave mucin molecules and therefore gut microbe-mediated mucin turnover is part of healthy gut function[13].

One consequence of fibre-deprived diets such as the WD is decreased abundance of fibre-degrading microbes, and their beneficial metabolites, including short chain fatty acids (SCFAs) such as butyrate, acetate and propionate. SCFAs are key microbial metabolites involved in immune regulation and gut barrier integrity. While butyrate and propionate are dominantly utilised locally in the gut or liver, acetate can readily be detected in systemic circulation suggesting that it could also modulate immune function at more distant sites. These SCFAs can bind to key receptors including GPR43 and GPR109A on intestinal epithelial cells, which promotes epithelial barrier repair and turnover via NLRP3 inflammasome activation[14,15]. Butyrate is also the primary energy source for epithelial cells and is vital in modulating host immune responses[14,16] and

*These authors contributed equally.

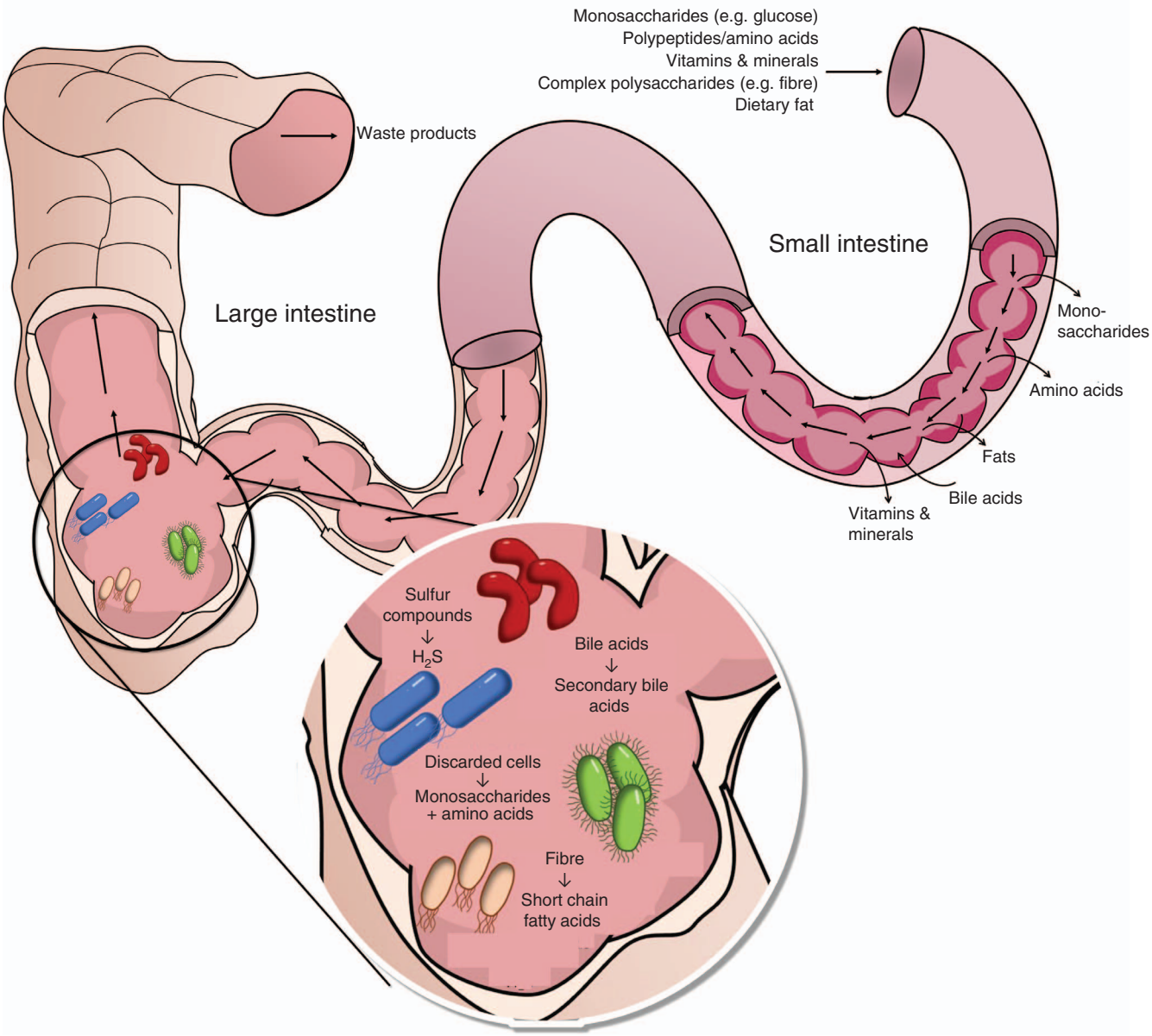

Figure 1. Digestive processes and microbial metabolism in the small and large intestine. Digestion and absorption of monosaccharides, amino acids and lipids occurs in the small intestine. Host-indigestible carbohydrates (fibre), along with unabsorbed nutrients and by-products of digestion pass into the large intestine where the majority of gut microbes are present. Microbial metabolic processes in the large bowel results in a variety of metabolites that can be beneficial or detrimental for gut health.

maintains the epithelial barrier by decreasing epithelial permeability through upregulating tight junction proteins (Figure 2), including zonula occludens protein 1 and members of the claudin protein family[17]. SCFAs can also promote the differentiation and accumulation of regulatory T-cells (Treg) in the gut, central to the maintenance of immune tolerance[18,19].

While fibre is the dominant dietary-derived nutrient source in the large intestine, some microbes are able to utilise glycoprotein-rich mucins as an alternative energy source[20], including *Akkermansia mucinophila* and members of the *Bacteroides* genus[13]. Mucin turnover is critical for maintaining intestinal integrity, although a tight balance between mucus degradation and renewal is required, with an essential role for mucin-degrading microbes. However, fibre-deprived environments select for microbes with the ability to utilise mucins, and can lead to excessive degradation of the mucus layer exposing the underlying epithelial cells to luminal antigen, promoting inflammatory responses (Figure 2). Furthermore, as mucin is an endogenous source of sulfur, an additional outcome of excessive mucin degradation is increased production of hydrogen sulfide (H_2S) by sulfate-reducing bacteria such as *Bilophila* spp. and *Desulfovibrio* spp. H_2S is a genotoxic compound that has been shown to damage DNA and trigger chromosomal instability[21].

Increased levels of primary bile acids are also associated with the WD, required for emulsification of dietary fat (Figure 1). While much of the bile acid pool is reabsorbed in the ileum, bile acids are subject to extensive microbial metabolism including deconjugation of amino acids taurine and glycine, and conversion to secondary bile acids (SBAs)[22]. Certain SBAs such as 3-oxolithocholic acid

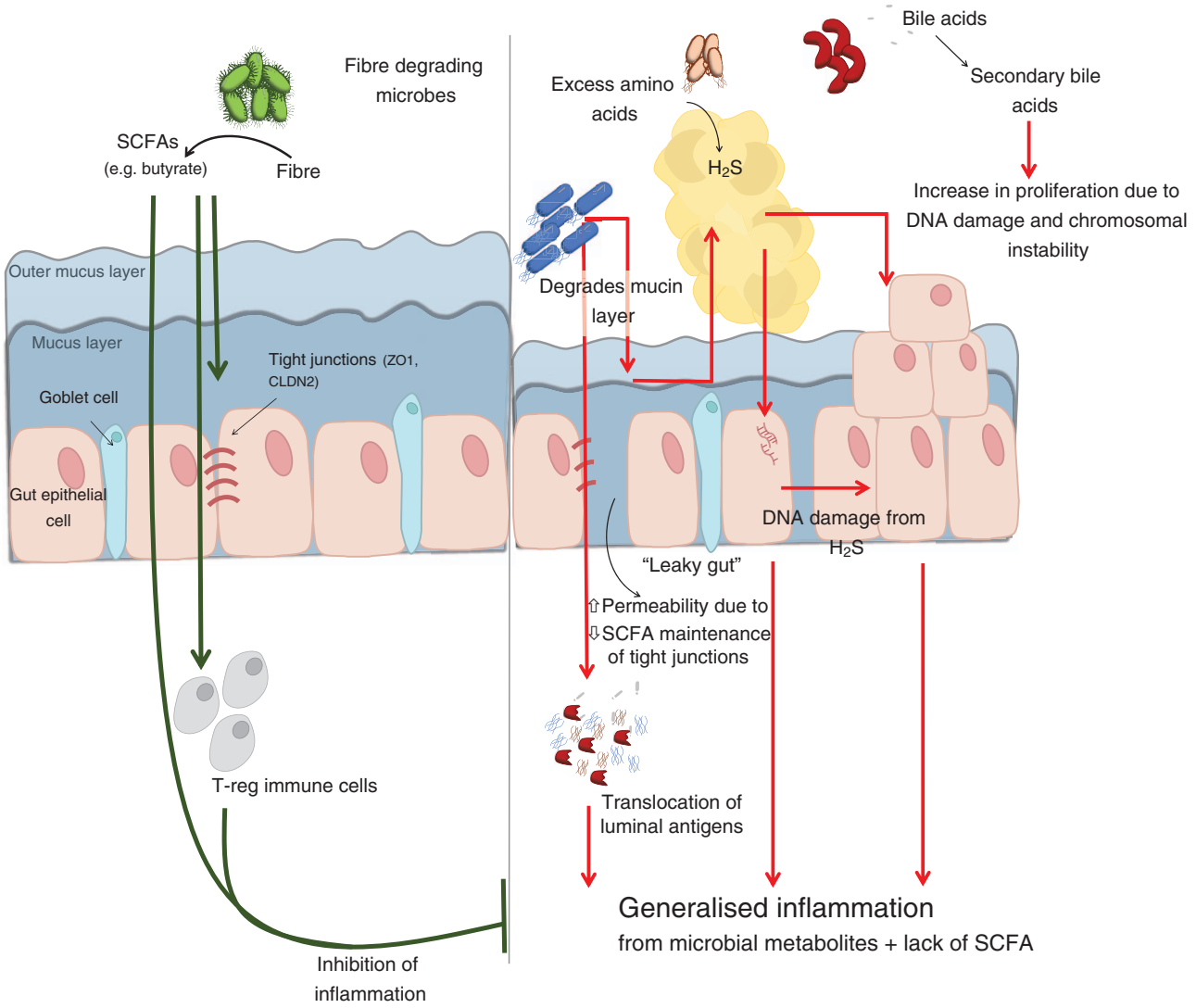

Figure 2. Dietary intake and nutrient availability shapes the balance between pro and anti-inflammatory properties of the gut microbiome. (A) In healthy, high fibre environment, microbes will degrade complex carbohydrates resulting in SCFA production. Healthy mucin turnover occurs through interaction with mucin degrading microbes. Optimal intestinal barrier function and immune regulation are favoured. (B) In diets lacking dietary fibre, and high in fats and/or protein, there is reduced production of beneficial short chain fatty acids, degradation of the mucin layer through excess microbial degradation, and production of potentially detrimental metabolites. This can result in increased permeability of the intestinal barrier leading to inflammation and excess proliferation of epithelial cells.

and isoallolithocholic acid have immunomodulatory properties, inhibiting the generation of T helper 17 cells and promoting the differentiation of regulatory T cells respectively[23]. The SBA 3β-hydroxydeoxycholic acid also shapes the gut immune response by inhibiting the ability of dendritic cells to activate adaptive immune cells, leading to an increase in regulatory T cells in the colon[24]. Activation of the bile acid receptor TGR5/GPBAR1 has also been shown to promote macrophage polarisation towards the anti-inflammatory M2 phenotype, and reduce the expression of inflammatory genes in a mouse model of colitis[25]. The immunoregulatory effects of these SBAs may be beneficial in mitigating inflammatory bowel diseases, and may help to prevent the development of colorectal cancer. However, other microbe-transformed bile acids such as deoxycholic and lithocholic acid have pro-carcinogenic properties (Figure 2) and predominantly act via the downregulation of p53, a tumour suppressor gene, and the generation of ROS to induce DNA damage and genomic instability, eventually resulting in increased cell proliferation[26,27]. Furthermore, during SBA production, sulfur-containing taurine is available for H_2S generation[22].

Therefore, the synergistic effect of the removal of fibre and high levels of saturated fat in the diet, as seen in the WD, can lead to reduced epithelial barrier function, erosion of the mucosal layer, inflammation and an increased susceptibility to luminal pathogens and carcinogens. The altered nutrient availability can also result in unfavourable gut microbiome compositions that have the potential to drive inflammation within the gut, therefore resulting in poor gut health.

In addition to inflammation-associated disorders of the gut such as IBD, these impacts on the gut epithelium are relevant to both local and systemic cancer outcomes. Locally, colorectal cancer (CRC) is linked to long term consumption of a WD[28]. CRC risk is determined by complex diet–microbe interactions, where the production of toxic microbial metabolites are capable of driving pro-carcinogenic

responses that transform the epithelium[29]. In addition, increased epithelial permeability permeability – often referred to as 'leaky gut' (Figure 2) – enables the translocation of luminal antigens across the epithelium, promoting a local inflammatory response, while disruption to the mucus layer exposes stem cells to microbial metabolites that promote cell replication[30]. The outcome is uncontrolled proliferation of epithelial cells, resulting in tumour formation. However, predicting which individuals are most at risk of CRC development remains a challenge and further understanding of individual microbiome profiles, and how these interact with dietary intake, is required.

More recently, systemic impacts of the gut microbiome in the context of cancer immunotherapy distal to the gut have been identified. Immunotherapy acts to induce the immune system to target and eliminate cancer cells and has been used in various cancers including melanoma, lung and renal tumours[31–33]. Emerging evidence indicates that higher fibre consumption is associated with improved response rates to therapy[34], and the microbiome represents a promising target to overcome therapeutic resistance[35] and reduce side-effects such as colitis[36]. While the specific taxa linking response to treatment across cohorts lack consensus[31–33], shared functional properties such as fibre fermentation and mucin turnover that support intestinal epithelial barrier integrity may be the underlying common features. However, whether fibre supplementation will be effective at modulating the microbiome in a feasible timeframe to improve treatment responses requires further investigation.

Although diet is well established to shape the composition of the microbiome, how an individual responds to a particular dietary intervention is dependent on the composition of an individual's baseline microbiome. For example, individuals have been shown to respond differently to supplementation with the same type of fibre[37,38]. This has been linked to interspecies competition and functional redundancy within microbiome[39]. Inter-individual variation therefore presents a significant challenge in terms of designing effective therapeutic dietary interventions, as variable responses dependent on the assemblage of the microbiome would be expected. Additionally, different types of fibre are known to have different prebiotic effects, for example, not all fibre sources are equally capable of stimulating SCFA production[40,41]. Given the impact of inter-individual variation impact of an individual's baseline microbiome a 'one-size fits all' approach will likely be ineffective, rather more personalised approaches will be necessary to enhance the reproducibility and success of nutritional interventions in the clinic.

While a significant body of research has emerged in this area, there remains much to be understood. What mechanisms underly the various host–microbe interactions at the epithelial interface, in response to different diets, in a clinical setting? How do individual gut environments and microbial ecosystems impact responses to treatments such as immunotherapy? Can tools be created to predict and modulate these outcomes? Understanding these issues could enable the implementation of personalised medicine, where the individual's native microbiome, genetics, and dietary history could be considered prior to implementation of medical interventions, resulting in greater treatment effectiveness and fewer side-effects.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

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References

1. Statovci, D. *et al*. (2017) The impact of Western diet and nutrients on the microbiota and immune response at mucosal interfaces. *Front. Immunol.* **8**, 838. doi:10.3389/fimmu.2017.00838
2. Tayyem, R.F. *et al*. (2015) Macro- and micronutrients consumption and the risk for colorectal cancer among Jordanians. *Nutrients* **7**, 1769–1786. doi:10.3390/nu7031769
3. Chun, Y.J. *et al*. (2015) Associations of colorectal cancer incidence with nutrient and food group intakes in Korean adults: a case-control study. *Clin. Nutr. Res.* **4**, 110–123. doi:10.7762/cnr.2015.4.2.110
4. Liu, L. *et al*. (2011) Is dietary fat associated with the risk of colorectal cancer? A meta-analysis of 13 prospective cohort studies. *Eur. J. Nutr.* **50**, 173–184. doi:10.1007/s00394-010-0128-5
5. Sonnenburg, J.L. and Backhed, F. (2016) Diet-microbiota interactions as moderators of human metabolism. *Nature* **535**, 56–64. doi:10.1038/nature18846
6. Gibson, P.R. and Shepherd, S.J. (2005) Personal view: food for thought–western lifestyle and susceptibility to Crohn's disease. The FODMAP hypothesis. *Aliment. Pharmacol. Ther.* **21**, 1399–1409. doi:10.1111/j.1365-2036.2005.02506.x
7. Marsh, A. *et al*. (2016) Does a diet low in FODMAPs reduce symptoms associated with functional gastrointestinal disorders? A comprehensive systematic review and meta-analysis. *Eur. J. Nutr.* **55**, 897–906. doi:10.1007/s00394-015-0922-1
8. Koropatkin, N.M. *et al*. (2012) How glycan metabolism shapes the human gut microbiota. *Nat. Rev. Microbiol.* **10**, 323–335. doi:10.1038/nrmicro2746
9. Reese, A.T. *et al*. (2018) Microbial nitrogen limitation in the mammalian large intestine. *Nat. Microbiol.* **3**, 1441–1450. doi:10.1038/s41564-018-0267-7
10. Holmes, A.J. *et al*. (2017) Diet–microbiome interactions in health are controlled by intestinal nitrogen source constraints. *Cell Metab.* **25**, 140–151. doi:10.1016/j.cmet.2016.10.021
11. Hamilton, M.K. *et al*. (2015) Changes in intestinal barrier function and gut microbiota in high-fat diet-fed rats are dynamic and region dependent. *Am. J. Physiol. Gastrointest. Liver Physiol.* **308**, G840–G851. doi:10.1152/ajpgi.00029.2015
12. Odenwald, M.A. and Turner, J.R. (2017) The intestinal epithelial barrier: a therapeutic target? *Nat. Rev. Gastroenterol. Hepatol.* **14**, 9–21. doi:10.1038/nrgastro.2016.169
13. Tailford, L.E. *et al*. (2015) Mucin glycan foraging in the human gut microbiome. *Front. Genet.* **6**, 81. doi:10.3389/fgene.2015.00081
14. Singh, N. *et al*. (2014) Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* **40**, 128–139. doi:10.1016/j.immuni.2013.12.007
15. Macia, L. *et al*. (2015) Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat. Commun.* **6**, 6734. doi:10.1038/ncomms7734
16. Canani, R.B. *et al*. (2011) Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* **17**, 1519–1528. doi:10.3748/wjg.v17.i12.1519
17. Wang, K. *et al*. (2019) Claudin-7 downregulation induces metastasis and invasion in colorectal cancer via the promotion of epithelial-mesenchymal transition. *Biochem. Biophys. Res. Commun.* **508**, 797–804. doi:10.1016/j.bbrc.2018.10.049
18. Furusawa, Y. *et al*. (2013) Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* **504**, 446–450. doi:10.1038/nature12721

19. Smith, P.M. *et al*. (2013) The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569–573. doi:10.1126/science.1241165
20. Desai, M.S. *et al*. (2016) A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell* **167**, 1339–1353. e21. doi:10.1016/j.cell.2016.10.043
21. O'Keefe, S.J.D. (2016) Diet, microorganisms and their metabolites and colon cancer. *Nat. Rev. Gastroenterol. Hepatol.* **13**, 691–706. doi:10.1038/nrgastro.2016.165
22. Ridlon, J.M. *et al*. (2016) Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes* **7**, 22–39. doi:10.1080/19490976.2015.1127483
23. Hang, S. *et al*. (2019) Bile acid metabolites control TH17 and Treg cell differentiation. *Nature* **576**, 143–148. doi:10.1038/s41586-019-1785-z
24. Campbell, C. *et al*. (2020) Bacterial metabolism of bile acids promotes generation of peripheral regulatory T cells. *Nature* **581**, 475–479. doi:10.1038/s41586-020-2193-0
25. Biagioli, M. *et al*. (2017) The bile acid receptor GPBAR1 regulates the M1/M2 phenotype of intestinal macrophages and activation of GPBAR1 rescues mice from murine colitis. *J. Immunol.* **199**, 718–733. doi:10.4049/jimmunol.1700183
26. Bernstein, H. *et al*. (2005) Bile acids as carcinogens in human gastrointestinal cancers. *Mutat. Res. Rev. Mutat. Res.* **589**, 47–65. doi:10.1016/j.mrrev.2004.08.001
27. Cao, H. *et al*. (2014) The secondary bile acid, deoxycholate accelerates intestinal adenoma–adenocarcinoma sequence in Apc min/+ mice through enhancing Wnt signaling. **13**, 563–571.
28. O'Keefe, S.J.D. *et al*. (2015) Fat, fibre and cancer risk in African Americans and rural Africans. 6, 6342.
29. Gagnière, J. *et al*. (2016) Gut microbiota imbalance and colorectal cancer. *World J. Gastroenterol.* **22**, 501–518. doi:10.3748/wjg.v22.i2.501
30. Kaiko, G.E. *et al*. (2016) The colonic crypt protects stem cells from microbiota-derived metabolites. *Cell* **165**, 1708–1720. doi:10.1016/j.cell.2016.05.018
31. Gopalakrishnan, V. *et al*. (2018) Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* **359**, 97–103. doi:10.1126/science.aan4236
32. Matson, V. *et al*. (2018) The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* **359**, 104–108. doi:10.1126/science.aao3290
33. Routy, B. *et al*. (2018) Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* **359**, 91–97. doi:10.1126/science.aan3706
34. Spencer, C.N. *et al*. (2019) Abstract 2838: The gut microbiome (GM) and immunotherapy response are influenced by host lifestyle factors. *Cancer Res.* **79**, 2838.
35. Simpson, R.C. *et al*. (2021) Targeting the microbiome to overcome resistance. *Cancer Cell* **39**, 151–153. doi:10.1016/j.ccell.2021.01.016
36. Batten, M. *et al*. (2020) Gut microbiota predicts response and toxicity with neoadjuvant immunotherapy. *Cancer Res.* **80**, 5734.
37. Kovatcheva-Datchary, P. *et al*. (2015) Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of *Prevotella*. *Cell Metab.* **22**, 971–982. doi:10.1016/j.cmet.2015.10.001
38. Healey, G. *et al*. (2016) Influence of habitual dietary fibre intake on the responsiveness of the gut microbiota to a prebiotic: protocol for a randomised, double-blind, placebo-controlled, cross-over, single-centre study. *BMJ Open* **6**, e012504. doi:10.1136/bmjopen-2016-012504
39. Patnode, M.L. *et al*. (2019) Interspecies competition impacts targeted manipulation of human gut bacteria by fiber-derived glycans. *Cell* **179**, 59–73 e13. doi:10.1016/j.cell.2019.08.011
40. Walker, A.W. *et al*. (2011) Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J.* **5**, 220–230. doi:10.1038/ismej.2010.118
41. Baxter, N.T. *et al*. (2019) Dynamics of human gut microbiota and short-chain fatty acids in response to dietary interventions with three fermentable fibers. *MBio* **10**, e02566-18. doi:10.1128/mBio.02566-18

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Cervicovaginal microbiota and women's health outcomes

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Abstract. The human cervicovaginal microbiome has an important role in the health and homoeostasis of the female reproductive tract. A eubiotic microbiome is typically dominated with lactic acid producing bacteria and is categorised into five community state types. Issues arise when the microbiome becomes dysbiotic, with the microbial composition shifting to contain a greater relative abundance of strict and facultative anaerobes. This shift will lead to several adverse changes in the vaginal environment including compromised epithelial cells, cell death, inflammation, and greater susceptibility to infection. These changes are associated with various adverse outcomes including infections, preterm birth, and infertility. In this review, we discuss how the cervicovaginal microbiome influences these outcomes and possible future directions of treatment and research.

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Introduction

The human microbiome is a unique collection of microorganisms that colonises the body and has an important role in health and homeostasis. The cervicovaginal microbiome is particularly distinctive as it is frequently dominated by *Lactobacillus* with decreased diversity of other bacteria, unlike what is seen in other sites such as the gut[1]. The cervicovaginal microbiome is extremely important to the host tissue as it maintains an acidic environment, preventing pathogenic colonisation, and modulates inflammation by cross-kingdom signalling[1]. Thus, the composition of the cervicovaginal microbiome plays an important role in health outcomes for women, particularly in relation to vaginal infection, pregnancy, and fertility.

The eubiotic microbiome

Early culture-based studies identified *Lactobacillus* as the dominant bacteria in the vaginal microbiome and recognised that it may play a key role in maintaining the health of the female reproductive tract[2]. Molecular-based techniques, including relatively recent next generation sequencing, have been used to obtain an in-depth understanding of vaginal flora and to classify microbiota into broad profiles termed community state types (CST)[3,4]. Four CSTs are dominated by a species of *Lactobacillus*; *Lactobacillus crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III) and *L. jensenii* (CST V). CST IV is characterised by various strict and facultative anaerobes and is typified by the absence of a dominant *Lactobacillus* species. The CSTs have varying levels of stability and transitions between CSTs are associated with composition, menstrual cycle, and sexual activity[4]. *Lactobacillus* produces lactic acid, maintaining vaginal pH at $\leq$4.5, promoting a selective environment for acid tolerant bacteria whilst suppressing pathogenic colonisation (Figure 1). Lactic acid has an immunomodulatory function, acting directly on epithelial cells to promote an anti-inflammatory response via the production of interleukin (IL)-1 receptor antagonist, as well as promoting the production of pro-inflammatory mediators and antimicrobial peptides (Figure 1)[5].

The dysbiotic microbiome

Dysbiosis is defined as a change in microbiota composition relative to the community of commensal bacteria seen in a healthy state[6]. There are no specific bacteria universally seen in dysbiosis but it is frequently associated with increased relative abundance of *Gardnerella, Prevotella*, and *Atopbium*[7,8]. This shift in composition results in a decrease in lactic acid, with an increase in short chain fatty acids, amines, and pH (Figure 2)[9]. Dysbiosis is also associated with several detrimental changes in the cervicovaginal environment including alterations in the cytoskeleton, increased cell death, an imbalance in the concentration of antimicrobial peptides and increased production of pro-inflammatory cytokines (Figure 2)[10]. These changes are thought to leave the tissue susceptible to infection and inflammation.

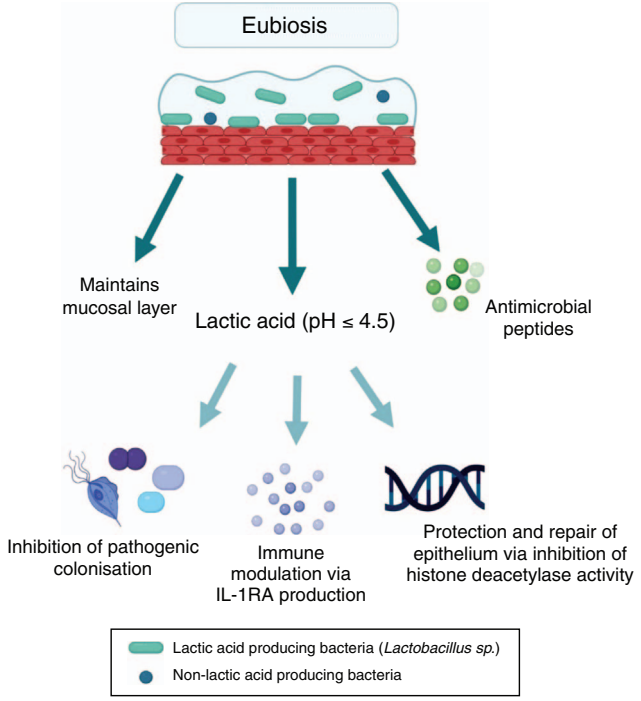

Figure 1. Eubiotic microbiome. Bacteria maintain the mucosal layer, release antimicrobial peptides, and lactic acid. Lactic acid lowers the pH, preventing pathogenic colonisation, and modulating the immune response, protecting the epithelial layer. Created with BioRender.com (https://biorender.com/).

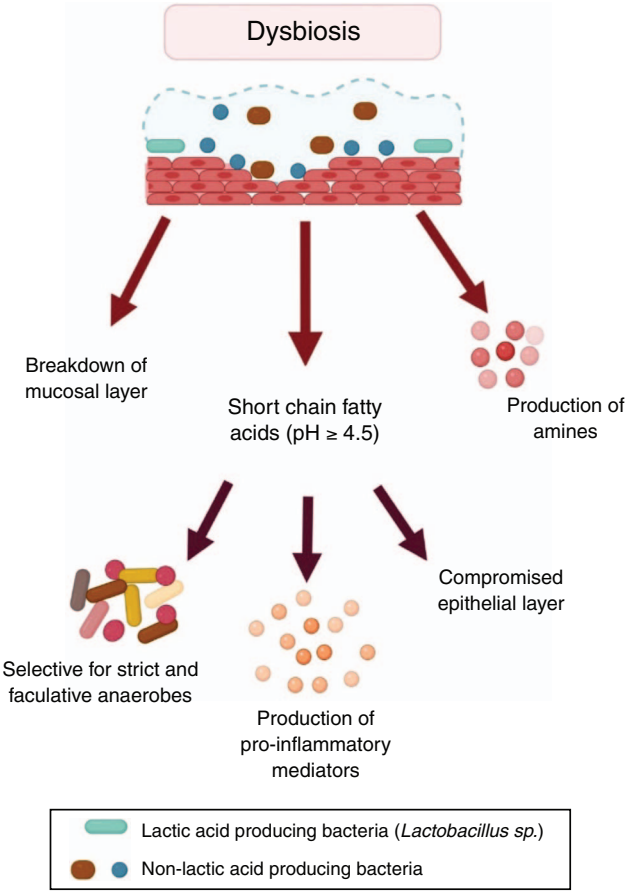

Figure 2. Dysbiotic microbiome. There is a breakdown in the mucosal layer, and the production of amines and short chain fatty acids, increasing the pH. This creates an environment selective for strict and facultative anaerobes, a pro-inflammatory response in the tissue, and compromises the epithelium. Created with BioRender.com (https://biorender.com/).

Bacterial vaginosis

Bacterial vaginosis (BV) is the most common vaginal infection, characterised by dysbiosis and the associated metabolomic changes. BV often is asymptomatic, but women may experience symptoms such as discoloured vaginal discharge, and a 'fishy' odour. The prevalence of BV is variable between different populations but worldwide prevalence is approximately 30%, with prevalence in Australia considerably lower at 4.7%[11]. Treatment with oral or intravaginal antibiotics is only recommend for women experiencing symptoms. However, after treatment, reoccurrence is common with up to 60-80% of women experiencing reoccurrence within 12 months after treatment[12]. Recent research has now shifted to investigating the variables associated with reoccurrence, specifically microbiota composition, to improve the treatment outcomes for women with BV[13].

Sexually transmitted infections

Dysbiosis of the cervicovaginal microbiota is known to increase the risk of acquiring a sexually transmitted infection (STI). Numerous longitudinal studies have determined that high microbiota diversity increases the risk of acquiring an infection[14]. A possible mechanism that increases susceptibility may be an inflammatory response to diverse bacteria. Gosmann *et al.*[15] investigated the association between the microbiome, inflammation, and human immunodeficiency virus (HIV)-acquisition in a prospective cohort study of South Africa women. They determined that women with polymicrobial microbiomes dominated with anaerobes, had increased activated mucosal $CD4^+$ T cells, and four-fold greater risk of HIV infection. They suggested that the target cells were responding to the microbial diversity, which in turn increase host susceptibility[15]. A similar response is also hypothesised to be involved in human papillomavirus infection, but is yet to be investigated[16]. Another mechanism involved in the susceptibility is the modulation of cellular functions. Ceccarani *et al.*[17] investigated the changes in the microbiome and metabolome during *Chlamydia* infection. In comparison to a healthy state, they showed clear changes in composition occurred during infection, specifically a decrease in lactic acid. Similarly, Edwards *et al.*[18] showed D (-) lactic acid produced by the microbiome may prevent cellular proliferation, protecting against *Chlamydia* infection. They suggested that a eubiotic microbiome modulates cell function preventing *Chlamydia* infection *in vitro*. These studies support that via direct metabolic profiles and cross talk involved in host cell responses, the cervicovaginal microbiome influences the risk of STI acquisition.

Pregnancy

The composition of the cervicovaginal microbiome has been associated with increased risk of adverse outcomes in pregnancy such as preterm birth. Preterm birth is defined as either a live or still birth after 20 weeks' gestation but before 37 weeks[19]. During pregnancy, hormonal changes alter the composition of the cervicovaginal microbiota resulting in an increased abundance of *Lactobacillus*. Several studies have shown that women with a diverse, non-*Lactobacillus* dominated microbiome are at a greater risk of preterm birth[20,21]. However, there is no defined profile of bacteria associated with adverse outcomes and results from each study greatly vary due to the population and study design. Kosti *et al*.[22] recently conducted meta-analysis to address these issues and created a microbial signature associated with preterm birth. They successfully identified a lack of *Lactobacillus* as a predictor of preterm birth, alongside several species that had been previously reported. Interestingly, they identified an association between preterm birth and the presence of *Olsenella* and *Clostridium sensu stricto*, which had not been previously reported[22]. Overall, these promising results show the potential for novel diagnostics that could guide interventions to improve pregnancy outcomes for women at risk.

Infertility treatment

Infertility is defined as the inability to attain a clinical pregnancy after 12 months of regular unprotected intercourse[19]. *In vitro* fertilisation (IVF) is now the most common procedure used to treat a range of infertility issues[23]. However, in Australia, the success rate of IVF procedures is reported as approximately 30% with little improvement over the past 5 years[24]. Poor outcomes of IVF have been associated with the composition of the cervicovaginal microbiome in several studies, although these studies often have a small sample size and mixed quality of methodologies. Initially Hyman *et al*.[20] associated diverse vaginal bacteria with poor IVF outcomes and suggested that the composition of the microbiome at the time of embryo transfer may be an important factor in the success of IVF treatment. Since this initial study there have been several others that have associated increased diversity of cervicovaginal microbiota and the presence of specific bacteria, with IVF failure[25–27]. However, there is no defined profile of microbiota associated with poor outcomes in IVF treatment, mostly due to the lack of larger studies. To understand the pathogenesis of this relationship, Fu *et al*.[28] conducted a study to assess changes in the microbiome and metabolome in association with the outcomes of IVF failure. They determined that there was a lack of key metabolites necessary for embryo development and implantation such as glycerophospholipids and benzopyran in those who experienced IVF failure, and in turn these metabolite differences were associated with different compositions of microbiota. While this study shows some promising results, the pathophysiology involved in this relationship is yet to be fully explored.

Future directions

It is clear the cervicovaginal microbiome plays a key role in health outcomes for women, with dysbiosis commonly observed in a range of adverse events. However, the mechanisms underlying these relationships are not well understood. Future microbiome and metabolome models will provide a method of representing these interactions *in vitro*. Delgado-Diaz *et al*.[29] used key metabolites associated with a *Lactobacillus* dominated microbiome and BV-associated microbiome to model the response of cells. This showed the immunomodulatory effect of lactic acid, but also showed that a lack of lactic acid and high concentrations of short chain fatty acids would stimulate increased production of pro-inflammatory cytokines. Thus, the approach of using an *in vitro* model is a promising method to better understand the microbiome and host cell interplay at a molecular level. Furthermore, large studies are necessary to determine predictive biomarkers of adverse outcomes, and to inform development of treatments such as probiotics for targeted treatment of the microbiome.

Conflicts of interest

The authors declare no conflicts of interest.

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References

1. García-Velasco, J.A. *et al*. (2020) The reproductive microbiome – clinical practice recommendations for fertility specialists. *Reprod. Biomed. Online* **41**, 443–453. doi:10.1016/j.rbmo.2020.06.014
2. Redondo-Lopez, V. *et al*. (1990) Emerging role of lactobacilli in the control and maintenance of the vaginal bacteria microflora. *Rev. Infect. Dis.* **12**, 856–872. doi:10.1093/clinids/12.5.856
3. Ravel, J. *et al*. (2011) Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. USA* **108**, 4680–4687. doi:10.1073/pnas.1002611107
4. Gajer, P. *et al*. (2012) Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* **4**, 132ra52. doi:10.1126/scitranslmed.3003605
5. Hearps, A.C. *et al*. (2017) Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. *Mucosal Immunol.* **10**, 1480–1490. doi:10.1038/mi.2017.27
6. Petersen, C. and Round, J.L. (2014) Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol.* **16**, 1024–1033. doi:10.1111/cmi.12308
7. Fredricks, D.N. *et al*. (2005) Molecular identification of bacteria associated with bacterial vaginosis. *N. Engl. J. Med.* **353**, 1899–1911. doi:10.1056/NEJMoa043802

8. Srinivasan, S. *et al*. (2012) Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* **7**, e37818. doi:10.1371/journal.pone.0037818
9. Vitali, B. *et al*. (2015) Vaginal microbiome and metabolome highlight specific signatures of bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **34**, 2367–2376. doi:10.1007/s10096-015-2490-y
10. Borgdorff, H. *et al*. (2016) Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. *Mucosal Immunol.* **9**, 621–633. doi:10.1038/mi.2015.86
11. Fethers, K.A. *et al*. (2009) Early sexual experiences and risk factors for bacterial vaginosis. *JID* **200**, 1662–1670. doi:10.1086/648092
12. Coudray, M.S. and Madhivanan, P. (2020) Bacterial vaginosis—a brief synopsis of the literature. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **245**, 143–148. doi:10.1016/j.ejogrb.2019.12.035
13. Xiao, B. *et al*. (2019) Association analysis on recurrence of bacterial vaginosis revealed microbes and clinical variables important for treatment outcome. *Front. Cell. Infect. Microbiol.* **9**, 189. doi:10.3389/fcimb.2019.00189
14. Brotman, R.M. (2011) Vaginal microbiome and sexually transmitted infections: an epidemiologic perspective. *J. Clin. Invest.* **121**, 4610–4617. doi:10.1172/JCI57172
15. Gosmann, C. *et al*. (2017) Lactobacillus-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in young South African women. *Immunity* **46**, 29–37. doi:10.1016/j.immuni.2016.12.013
16. Torcia, M.G. (2019) Interplay among vaginal microbiome, immune response and sexually transmitted viral infections. *Int. J. Mol. Sci.* **20**, 266. doi:10.3390/ijms20020266
17. Ceccarani, C. *et al*. (2019) Diversity of vaginal microbiome and metabolome during genital infections. *Sci. Rep.* **9**, 14095. doi:10.1038/s41598-019-50410-x
18. Edwards, V.L. *et al*. (2019) The cervicovaginal microbiota-host interaction modulates *Chlamydia trachomatis* infection. *MBio* **10**, e01548-19. doi:10.1128/mBio.01548-19
19. Zegers-Hochschild, F. *et al*. (2009) International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. *Fertil. Steril.* **92**, 1520–1524. doi:10.1016/j.fertnstert.2009.09.009
20. Hyman, R.W. *et al*. (2014) Diversity of the vaginal microbiome correlates with preterm birth. *Reprod. Sci.* **21**, 32–40. doi:10.1177/1933719113488838
21. Stafford, G.P. *et al*. (2017) Spontaneous preterm birth is associated with differential expression of vaginal metabolites by lactobacilli-dominated microflora. *Front. Physiol.* **8**, 615. doi:10.3389/fphys.2017.00615
22. Kosti, I. *et al*. (2020) Meta-analysis of vaginal microbiome data provides new insights into preterm birth. *Front. Microbiol.* **11**, 476. doi:10.3389/fmicb.2020.00476
23. Eichenberg, C. (2020) *In vitro* fertilization (IVF) *J Repro Sci.* **1**.
24. Newman, J.E. *et al*. (2020) Assisted reproductive technology in Australia and New Zealand 2018, National Perinatal Epidemiology and Statistic Unit, Sydney.
25. Haahr, T. *et al*. (2016) Abnormal vaginal microbiota may be associated with poor reproductive outcomes: a prospective study in IVF patients. *Hum. Reprod.* **31**, 795–803. doi:10.1093/humrep/dew026
26. Kitaya, K. *et al*. (2019) Characterization of microbiota in endometrial fluid and vaginal secretions in infertile women with repeated implantation failure. *Mediators Inflamm.* **2019**, 4893437. doi:10.1155/2019/4893437
27. Kong, Y. *et al*. (2020) The disordered vaginal microbiota is a potential indicator for a higher failure of *in vitro* fertilisation. *Front. Med.* **7**, 217. doi:10.3389/fmed.2020.00217
28. Fu, M. *et al*. (2020) Alterations in vaginal microbiota and associated metabolome in women with recurrent implantation failure. *MBio* **11**, e03242-19. doi:10.1128/mBio.03242-19
29. Delgado-Diaz, D.J. *et al*. (2020) Distinct immune response elicited from cervicovaginal epithelial cells by lactic acid and short chain fatty acids associated with optimal and non-optimal and vaginal microbiota. *Front. Cell. Infect. Microbiol.* **9**, 446. doi:10.3389/fcimb.2019.00446

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Vaginal microbial profiling in a preterm birth high-risk cohort using shallow shotgun metagenomics

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Abstract. Preterm birth (PTB) is a significant health problem globally, with an estimate of 15 million cases annually. Approximately 10% of neonates born early will die prematurely, while a subset will develop severe life-long morbidities. Unfortunately, preterm birth's syndromic nature has evaded prevention strategies, and it continues to impose a high burden on healthcare systems and families. The role of vaginal bacteria in triggering biomolecular causes of PTB has been recognised for years. However, translating this knowledge to practical diagnostic and therapeutic strategies has remained elusive. New techniques in high-throughput sequencing have improved our understanding of the nature and role of the vaginal microbiome during pregnancy. Several multi-ethnic and multi-geographical studies into the vaginal microbiome have identified five distinct bacterial profiles termed community state types (CSTs), one of which is positively associated with dysbiosis and increased risk of PTB. In a small pilot study of first-trimester vaginal microbial DNA obtained from pregnant women at high-risk of PTB, we compared the CST profiles generated using standard 16S amplicon sequencing with shallow shotgun metagenomics (SSM). Both methods identified the presence of the five CSTs as has been reported previously, although the metagenomic data showed greater taxonomic resolution and more accurate CST assignation. These findings suggest that SSM is a cost-effective and potentially superior alternative to 16S sequencing for vaginal microbiome analysis.

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Introduction

Preterm birth (PTB), defined by the World Health Organization as all deliveries between 20–37 weeks of completed gestation, is a complex syndrome. The condition is divided into four groups based on gestational age (GA) at of birth: extreme PTB (<28 GA), very PTB (28-32 GA), moderate PTB (32-34 GA) and late PTB (34-37 GA)[1]. PTB impacts the lives of 15 million families annually, with an approximate 10% mortality rate in the first month after delivery[2]. Despite advances in neonatal care and improved survival and reduced morbidity, preterm infants are at high risk of developing metabolic disorders and debilitating neurological conditions, such as blindness, deafness, neurodevelopmental delays, and behavioural issues well into adulthood[3]. A recent meta-analysis of PTB hospitalisation costs in the US, Canada, and The Netherlands reported that the individual healthcare costs for extreme PTB were between $111 152–$576 972 per delivery[4].

PTB is a syndrome that is both difficult to predict and to prevent[5]. Multiple methods and approaches for PTB prediction have been developed and evaluated, with varying success[6–9]. Similarly, preventative treatments are limited and lack the required efficacy, applicability and precision. Women identified as at high risk of PTB (typically due to either a previous PTB and/or a short cervical length defined as <25 mm) typically receive one of two clinically-recommended preventive interventions at the discretion of the treating obstetrician, namely exogenous progesterone (vaginally, orally or intramuscularly) or cervical cerclage surgery[10].

A meta-analysis from 2018 with large high-risk pregnancies cohorts report that vaginal progesterone (VP) use resulted in a pooled relative risk ratio (RR) of 0.29–0.68, while cervical cerclage had a RR of 0.64–0.70[11]. The effectiveness of VP appears to be particularly robust in high-risk women with short cervical length (<25 mm), as has been recently demonstrated in the EPPPIC meta-analysis[12].

PTB has long been known to be associated with ascending intrauterine infections originating from a dysbiotic (sub-optimal) lower vaginal tract microbiome[5,13,14]. In 2–27% of pregnant women, the microbiome composition shifts to an increase in species

diversity, leading to a dysbiotic vaginal microbiome associated with a disease state. Several studies have now show that an increase in bacterial diversity is linked to reproductive tract inflammation and increased risk of PTB[15–19]. Despite numerous studies investigating the predictive usefulness of vaginal microbiome analysis, the diagnostic utility of this approach remains elusive. In a recent large study of low-risk Australian women, a high-risk microbial profile in the 2nd trimester was identified based on the presence/absence and combinations of known bacterial species[18]. Notwithstanding this study's clinical relevance to PTB management, it is important to point out that this particular work is based on analysis of a selected number of risk-associated bacteria, not the entire microbiome *per se*.

In 2011, Ravel and colleagues classified the vaginal microbiome of healthy reproductive-age women into five distinct community state types (CST), conditional on the dominance of one of four *Lactobacillus* spp. or lack thereof. CST-I is dominated by *Lactobacillus crispatus*, CST-II by *Lactobacillus gasseri*, CST-III by *Lactobacillus iners,* CST-IV by diverse anaerobic bacteria resembling the clinically diagnosed condition of bacterial vaginosis (BV), and CST-V by *Lactobacillus jensenii*[20]. The robustness of CST classifications has since been confirmed in many human microbiome studies, regardless of ethnicity, geographical location or sequencing methodology[21].

More recently, the Ravel laboratory developed the tool 'VALENCIA' (VAginaL community state typE Nearest CentroId clAssifier), which unbiasedly affirmed the presence of the original broad five CST profiles while defining an additional set of 13 subCST groups[22]. Importantly, VALENCIA links CST profiles with clinical descriptors across multiple ethnicities, plus provides researchers with the ability to accurately differentiate between known subtypes of CST-IV. The new CST-IV classification now takes into account the presence and abundance of *Lactobacillus* spp. and the following clinically essential bacteria: *Gardnerella vaginalis, Bifidobacterium* spp., *Atopobium vaginae, Prevotella* spp., *Enterococcus* spp., *Streptococcus* spp., and *Staphylococcus* spp.[22].

Notably, in the context of the vaginal microbiome, studies have shown that CST-IV and CST-III dominance early in pregnancy increase the risk of PTB[18,23], and it is now believed that microbial diversity assessment and CST profiling may help screen women for PTB risk[17]. Despite solid evidence associating PTB with CST suboptimal profiles, it is worth mentioning that most of the evidence was from studies with a predominantly Caucasian cohort. In African American cohorts, the associated significance was weak or disappeared altogether[24].

With the acceptance of ethnicity as a significant confounding factor, we know that vaginal microbiome dysbiosis is a substantial risk factor for uterine ascending infection, which has been causally linked to up to 40% of all preterm births[5]. However, the techniques used to generate microbiome data are often constrained by limited financial and bioinformatic resources, limiting their clinical and/or diagnostic utility. Therefore, employing methods that increase taxonomic resolution at a reasonable cost have the potential to enable CST profiling to be conducted for PTB risk prediction and treatment in high-risk pregnancies, as well as increasing the accuracy and resolution of the data.

Presently, the vaginal microbiome is typically studied via two DNA-based approaches and one RNA-based strategy: metabarcoding (DNA), metagenomics (DNA) and, to a lesser extent, metatranscriptomics (RNA, not discussed further)[25]. Metabarcoding (also known as metataxonomics or amplicon sequencing) is the most commonly used technique for microbiome analysis, partly due to its simplicity, but primarily because of the low cost (typically <$100 per sample) and well established analysis pipelines (e.g. USEARCH/DADA2). Amplicon sequencing involves the PCR amplification of a small hypervariable region or regions (250–500 bp) of the taxonomically informative 16S rRNA gene expressed in all bacterial species. Typically, microbiome specialists would design primers that can amplify a set of variable regions that allow the taxonomic discrimination and identification of bacterial genera – in some cases to the species level; this is necessary for CST profiling, although bias can be introduced through primer design, the selected 16S rRNA gene region and its coverage[25]. To eliminate obvious bias, primers may need to be redesigned to increase the species detection within the same taxonomic kingdom, or if separate domains are to be targeted, such as when characterising the prokaryotes, fungi and microeukaryotes communities present in the human vaginal tracts[25,26].

In contrast, metagenomics or shotgun sequencing has significant advantages over amplicon sequencing. It can remove detection bias by sequencing all DNA present in a sample, providing taxonomy to strain-level accuracy. Furthermore, it provides the researcher with the ability to assess metabolic functional potential of the genomes by conducting pathway analysis based on the sequenced genes. Although standard shotgun sequencing has advantages over amplicon sequencing, it carries some critical disadvantages: (1) the amount of DNA required is at least 1 µg; (2) analysis is expensive ($500–$1000 each); and (3) there is a requirement to have access to specialist bioinformatics resources and high-performance computing[27].

We have recently completed a pilot study assessing the taxonomic resolution resulting from a recent methodological advance in metagenomic analysis called *shallow shotgun metagenomics* (SSM)[28,29]. In SSM a sample is typically sequenced to a depth <1 million reads, which is an order of magnitude or more lower than the depth expected in a standard metagenomics study (depth between 10 million to 2.5 billion reads)[29]. The reduction in sequencing depth reduces the cost of SSM to those similar to amplicon sequencing, while retaining broad taxonomic coverage at higher taxonomic resolution with functional genetic information. Hillman and colleagues recently showed that a sequencing depth as low as 100 000 reads can mirror >90% of the alpha diversity and gene functional capacity relative to that mapped by ultra-deep metagenomics[28].

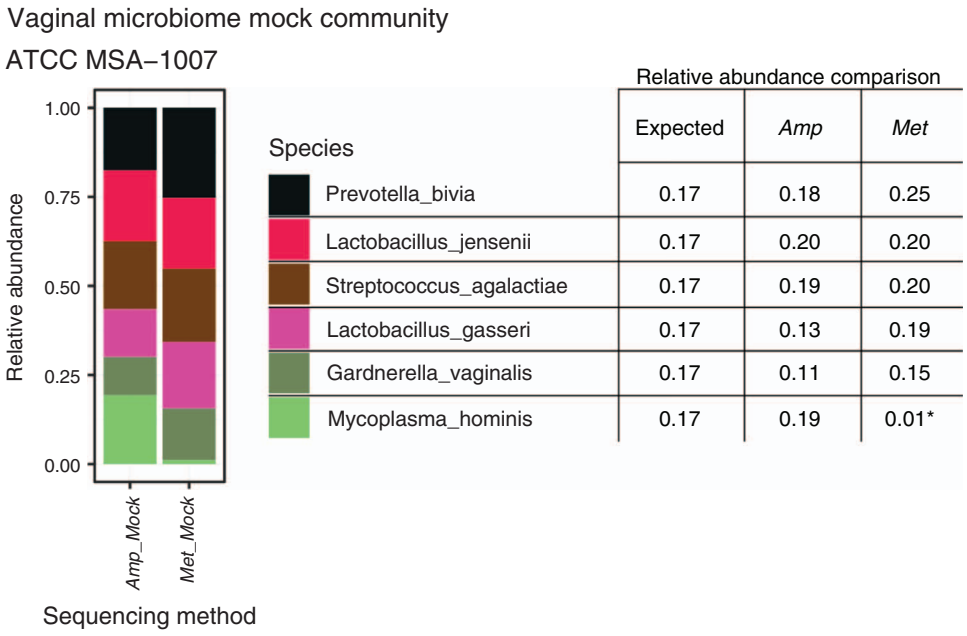
These attributes make SSM ideal for the study of microbiomes in large, longitudinal cohorts.

SSM also has two important practical limitations. Firstly, if a sample type contains a very high host:microbe DNA ratio, such as found in blood or tissue biopsies, then SSM may not be the method of choice, because the dominance of host DNA will swamp the reads assigned to microbes and low abundance species may be missed. Secondly, there are bioinformatic constraints, as most available tools are not designed to meet the particular requirements of SSM-generated data; this can result in the generation of false positives and negatives. Additionally, the entire metagenomics field is limited by the availability of high-quality genome databases due to the infancy of this field. Thus, rare or non-sequenced organisms are reported as negative or unassigned, potentially losing important taxonomic information and compromising interpretation. Although these points are all important limitations to consider in study design, in some microbiomes such as the skin or the vaginal microbiome (our research area) that contain a higher host DNA but low-to-medium biomass, SSM may still offer significant advantages due to the medical importance of identifying bacteria, fungi, viruses and micro-eukaryotes to species or strain resolution, which is not provided by amplicon sequencing.

In this study, we compared the bacterial taxonomic profile of SSM to standard 16S amplicon sequence in the context of the vaginal microbiota. The comparison was made using two sample sets: (1) a mock vaginal community consisting of six vaginal bacterial species with an even abundance of 16.7% to validate the robustness of the pipeline; and (2) DNA from 22 high vaginal swabs collected from women at high risk of PTB during their first trimester in Perth, Western Australia; the swabs were obtained from the Western Australian Pregnancy Biobank, with informed consent and institutional ethical approval. Our swabs yielded DNA concentrations between 1–40 ng/µL; two samples and the negative controls did not have enough DNA for sequencing, and thus were eliminated from analysis. The host DNA in the remaining 20 samples acquired on average 89% of the MiSeq Illumina sequenced reads, leaving only 2.1 million reads for the analysis of 20 samples (plus a mock community control).

Mock community analysis

First, we gauged the performance of our methods using the American Type Culture Collection (ATCC) standardized even abundance vaginal bacteria mock community (ATCC MSA-1007 medically relevant species). The sequencing comparison yielded a highly correlative bacterial composition (Figure 1). The data generated by amplicon sequencing (using primers targeting the v4 16S rRNA gene region) *vs*. SSM showed excellent taxonomic agreement, although there were some minor differences in relative abundance. However, it is worth mentioning that the (515f/806r) v4 primers used here were designed to enable detection of all six species and thus would be expected to amplify them preferentially. *Mycoplasma hominis* was markedly underrepresented in the *Met* (metagenomics) group where it represented only 1% of total species, while in the *Amp* (amplicon) group it was detected at 19% – very similar to the expected 16.7% in the mock community. We attribute this discrepancy in the *Met* group to the unavoidable stochasticity/compositionality introduced during sequencing, where the abundance of a species can be heavily skewed at random. Additionally, in this study we applied a completely PCR-free library preparation method to avoid amplification bias; however, this approach required a considerable amount (>100–1000 ng total) of starting genomic DNA, more than that provided with the ATCC



Species	Relative abundance comparison		
	Expected	*Amp*	*Met*
Prevotella_bivia	0.17	0.18	0.25
Lactobacillus_jensenii	0.17	0.20	0.20
Streptococcus_agalactiae	0.17	0.19	0.20
Lactobacillus_gasseri	0.17	0.13	0.19
Gardnerella_vaginalis	0.17	0.11	0.15
Mycoplasma_hominis	0.17	0.19	0.01*

Figure 1. Mock community bacterial species relative abundance differences between amplicon (*Amp*) and SSM (*Met*) sequencing methods. The table on the right corresponds to the relative abundance on a scale from 0–1 (rounded to 2 decimal places).

product (4 ng/µL). This does not offer a full explanation as to why the rest of the species in the mock community were not also detected at lower proportions than expected. We believe the difference is most likely driven by the fundamentally different genome-based reference database and tool used to assign taxonomy in SSM compared to the widely used extensive options available for bacterial amplicon sequencing. Interestingly, other studies comparing the outcome of mock communities and metagenomics also showed that amplicon sequencing seem to provide closer compositional agreement[30]. Importantly, this artificial situation would be unlikely to be replicated in a real-life analysis of complex, natural bacterial communities.

Vaginal swab analysis

Although the amplicon method showed considerable agreement on the taxonomic assignments of mock species, the SSM approach when applied to vaginal samples provided a species or strain level taxonomic assignation with high confidence as required for accurate vaginal CST determination. Figure 2 shows the relative abundance of the top 30 species in the 20 vaginal samples according to the two methods. While there was general agreement in the relative abundance of the most common species, several less abundant species were absent in the *Amp* group (e.g. *Neisseria gonorrhoeae)*. In addition, amplicon sequencing could not resolve the genus *Bifidobacterium* to species level, while SSM identified the species as *B. longum.* We also found that *L. iners* abundance was overrepresented in amplicon sequencing profiles. In contrast, SSM was able to resolve the same samples to either *L. jensenii or L. ultunensis* dominance. Enrichment of *L. iners* detection in the *Amp* group can be explained by preferential primer amplification.

As shown by amplicon sequencing, taxonomic uncertainty can be problematic to vaginal microbiome profiling, because it can distort the accurate picture of community composition and structure. In our analysis of CST profiles, we identified that these inaccuracies can result in CST-V or CST-IV being wrongly labelled as CST-III. This was evident in the sample from one patient (M65), whose profile was dominated by *L. ultunensis* as detected by SSM, but designated CST-III by amplicon sequencing (refer to Figure 3).

Although the detection of atypical CST types such as those dominated by species *L. ultunensis/amylovorus* posed a challenge during the allocation of CSTs, the fact that *Gardnerella vaginalis* seems to co-exist in these atypical communities prompted us to

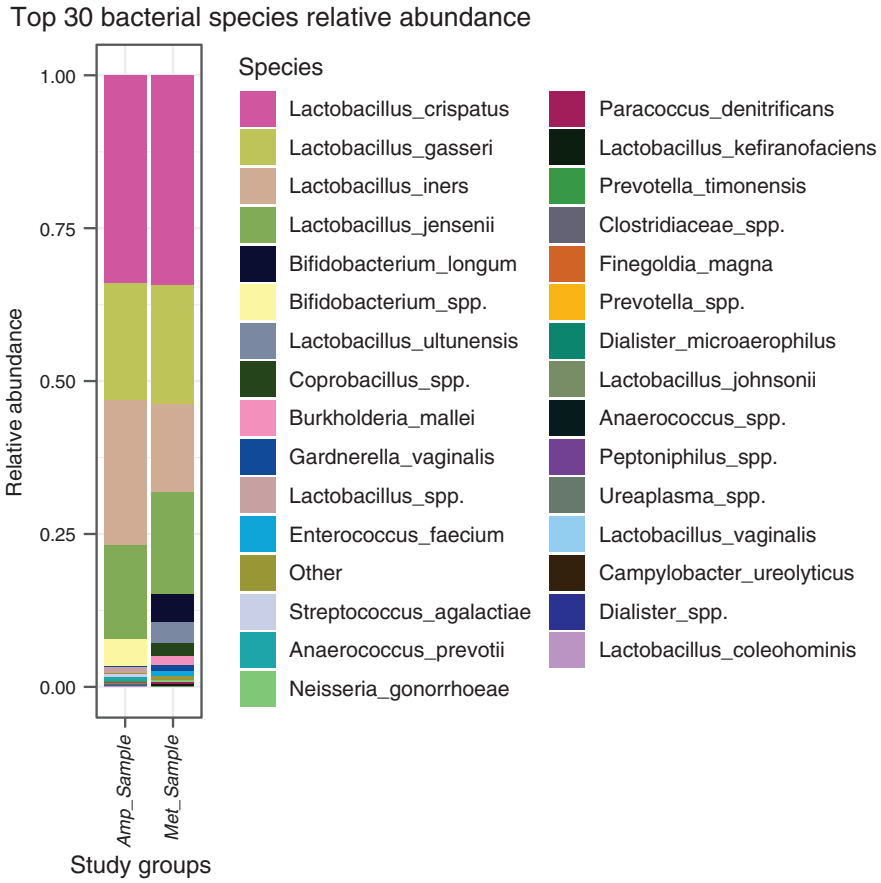


Figure 2. Shallow shotgun metagenomics (*Met*) provides greater taxonomic resolution than amplicon sequencing (*Amp*). The figure shows the aggregated species level taxonomy of all 20 samples for both *Met* and *Amp* methods. The bar plot shows the relative species abundance of patient vaginal samples resulting from the two methods. The X-axis shows the comparison groups with a prefix denoting the microbial survey approach used. Y-axis represents the relative abundance of sequencing reads assigned to each species. The legend on the right shows the top 30 bacterial species ordered according to the read abundance. Species with abundance smaller than the top 30 are grouped under 'Other'.

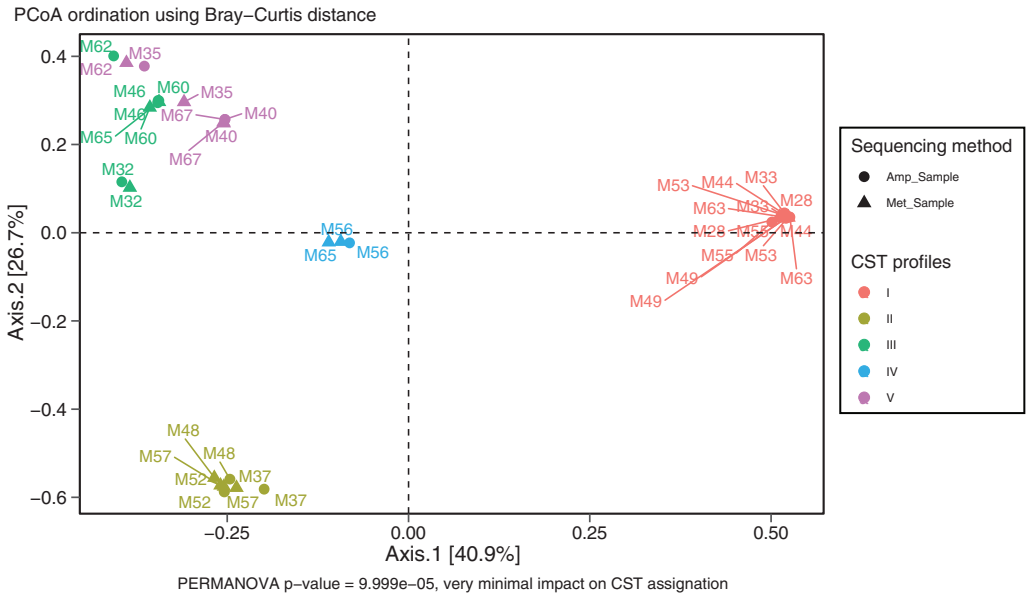


Figure 3. PCoA ordination plot generated using the Bray-Curtis dissimilarity index. The distance between samples represents the collective bacterial composition difference, which also differentiates the CST profiles of the cohort. Circles represent the *Amp* group and triangles are the *Met* samples. Samples are coloured according to the CST type they have been assigned by the respective method and labelled by the participant ID. The samples group into 5 CST clusters (*P*-value < 0.05) irrespective of sequencing method.

allocate them to CST-IV (mixture of facultative anaerobes with a moderate abundance of *G. vaginalis*). We took this approach to help in differentiating the atypical group dominant samples from other *Lactobacillus* dominated CST types commonly associated with vaginal microbial health. Although amplicon sequencing generates considerably lower taxonomic resolution than SSM, we believe it remains helpful as a tool for vaginal microbiome characterisation because it can broadly differentiate between CST types on *Lactobacillus* spp. dominance. Nonetheless, this comparison highlights the limitations of using amplicon sequencing in accurately distinguishing between closely related CST profiles such as those dominated by the *Lactobacillus* genus.

In conclusion, our pilot study suggests that shallow shotgun metagenomics is a superior method compared to amplicon sequencing in the context of species-level vaginal microbiome characterisation related to health and disease. Importantly, while standard (deep) metagenomics is cost-prohibitive for large studies, in this pilot study we show that the benefits associated with sequencing all DNA in a sample can be achieved at costs similar to amplicon sequencing. Our study also suggests that the vaginal microbiome data and CST demographics generated by high-resolution shotgun metagenomics may need to be re-examined in the context of microbial health and disease risk. Our follow-up work intends to improve our microbiome data accuracy and confidence by complementing shallow metagenomics laboratory workflow with a site-specific, multi-kingdom reference database combined with alternative bioinformatics algorithms.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of funding

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References

1. Blencowe, H. *et al.* (2013) Born too soon: the global epidemiology of 15 million preterm births. *Reprod. Health* **10**, S2. doi:10.1186/1742-4755-10-S1-S2
2. Chang, H.H. *et al.* (2014) Preventing preterm births: analysis of trends and potential reductions with interventions in 39 countries with very high human development index. *Obstet. Anesthes. Dig.* **34**, 16–17. doi:10.1097/01.aoa.0000443352.25554.e1
3. Hee Chung, E. *et al.* (2020) Neurodevelopmental outcomes of preterm infants: a recent literature review. *Transl. Pediatr.* **9**, S3–S8. doi:10.21037/tp.2019.09.10
4. Petrou, S. *et al.* (2019) Economic consequences of preterm birth: a systematic review of the recent literature (2009–2017). *Arch. Dis. Child.* **104**, 456. doi:10.1136/archdischild-2018-315778
5. Keelan, J.A. and Newnham, J.P. (2017) Recent advances in the prevention of preterm birth. *F1000Res.* **6**, 1139–1150. doi:10.12688/f1000research.11385.1
6. Guzman, E.R. *et al.* (2001) A comparison of sonographic cervical parameters in predicting spontaneous preterm birth in high-risk singleton gestations. *Ultrasound Obstet. Gynecol.* **18**, 204–210. doi:10.1046/j.0960-7692.2001.00526.x
7. Crane, J.M.G. and Hutchens, D. (2008) Transvaginal sonographic measurement of cervical length to predict preterm birth in asymptomatic women at increased risk: a systematic review. *Ultrasound Obstet. Gynecol.* **31**, 579–587. doi:10.1002/uog.5323
8. Iams, J.D. *et al.* (1996) The length of the cervix and the risk of spontaneous premature delivery. *N. Engl. J. Med.* **334**, 567–572. doi:10.1056/NEJM199602293340904
9. Cook, J. *et al.* (2019) First trimester circulating MicroRNA Biomarkers predictive of subsequent preterm delivery and cervical shortening. *Sci. Rep.* **9**, 5861. doi:10.1038/s41598-019-42166-1
10. Di Renzo, G.C. *et al.* (2011) Guidelines for the management of spontaneous preterm labor: identification of spontaneous preterm labor, diagnosis of preterm premature rupture of membranes, and preventive tools for preterm birth. *J. Matern. Fetal Neonatal Med.* **24**, 659–667. doi:10.3109/14767058.2011.553694

11. Conde-Agudelo, A. *et al*. (2018) Vaginal progesterone is as effective as cervical cerclage to prevent preterm birth in women with a singleton gestation, previous spontaneous preterm birth, and a short cervix: updated indirect comparison meta-analysis. *Am. J. Obstet. Gynecol.* **219**, 10–25. doi:10.1016/j.ajog.2018.03.028
12. Stewart, L.A. *et al*. (2021) Evaluating Progestogens for Preventing Preterm birth International Collaborative (EPPPIC): meta-analysis of individual participant data from randomised controlled trials. *Lancet* **397**, 1183–1194. doi:10.1016/S0140-6736(21)00217-8
13. Lamont, R.F. (2015) Advances in the prevention of infection-related preterm birth. *Front. Immunol.* **6**, 566. doi:10.3389/fimmu.2015.00566
14. Gravett, M.G. *et al*. (1986) Preterm labor associated with subclinical amniotic fluid infection and with bacterial vaginosis. *Obstet. Gynecol.* **67**, 229–237. doi:10.1097/00006250-198602000-00013
15. Kindinger, L.M. *et al*. (2016) Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage. *Sci. Transl. Med.* **8**, doi:10.1126/scitranslmed.aag1026
16. Fang, J. *et al*. (2020) Association of the vaginal microbiota with pregnancy outcomes in Chinese women after cervical cerclage. *Reprod. Biomed. Online* **41**, 698–706. doi:10.1016/j.rbmo.2020.06.016
17. Fettweis, J.M. *et al*. (2019) The vaginal microbiome and preterm birth. *Nat. Med.* **25**, 1012–1021. doi:10.1038/s41591-019-0450-2
18. Payne, M.S. *et al*. (2021) A specific bacterial DNA signature in the vagina of Australian women in midpregnancy predicts high risk of spontaneous preterm birth (the Predict1000 study). *Am. J. Obstet. Gynecol.* **224**, 206.e1–206.e23. doi:10.1016/j.ajog.2020.08.034
19. Freitas, A.C. *et al*. (2018) Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. *Microbiome* **6**, 117. doi:10.1186/s40168-018-0502-8
20. Ravel, J. *et al*. (2011) Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. USA* **108**, 4680–4687. doi:10.1073/pnas.1002611107
21. Ma, B. *et al*. (2020) A comprehensive non-redundant gene catalog reveals extensive within-community intraspecies diversity in the human vagina. *Nat. Commun.* **11**, 940. doi:10.1038/s41467-020-14677-3
22. France, M.T. *et al*. (2020) VALENCIA: a nearest centroid classification method for vaginal microbial communities based on composition. *Microbiome* **8**, 166. doi:10.1186/s40168-020-00934-6
23. Haque, M.M. *et al*. (2017) First-trimester vaginal microbiome diversity: a potential indicator of preterm delivery risk. *Sci. Rep.* **7**, 16145–16155. doi:10.1038/s41598-017-16352-y
24. Wells, J.S. *et al*. (2020) The vaginal microbiome in U.S. black women: a systematic review. *J. Womens Health (Larchmt.)* **29**, 362–375. doi:10.1089/jwh.2019.7717
25. Berman, H.L. *et al*. (2020) Understanding and interpreting community sequencing measurements of the vaginal microbiome. *BJOG* **127**, 139–146. doi:10.1111/1471-0528.15978
26. Hugerth, L.W. and Andersson, A.F. (2017) Analysing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. *Front. Microbiol.* **8**, 1561. doi:10.3389/fmicb.2017.01561
27. Boers, S.A. *et al*. (2019) Understanding and overcoming the pitfalls and biases of next-generation sequencing (NGS) methods for use in the routine clinical microbiological diagnostic laboratory. *Eur. J. Clin. Microbiol. Infect. Dis.* **38**, 1059–1070. doi:10.1007/s10096-019-03520-3
28. Hillmann, B. *et al*. (2018) Evaluating the information content of shallow shotgun metagenomics. *mSystems* **3**, e00069-18. doi:10.1128/mSystems.00069-18
29. Santiago-Rodriguez, T.M. *et al*. (2020) Metagenomic information recovery from human stool samples is influenced by sequencing depth and profiling method. *Genes (Basel)* **11**, 1380. doi:10.3390/genes11111380
30. Rausch, P. *et al*. (2019) Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome* **7**, 133. doi:10.1186/s40168-019-0743-1

Biographies

Alishum Ali is a Research Officer at Fiona Stanley Hospital Department of Neonatology and PhD student at Curtin University, with over 10 years' experience in molecular diagnostics. His PhD project aims to shed light on the links between extreme prematurity and vaginal microbiome and inflammation through the application of precision medicine principles and multi-omic analytical strategies. Ultimately, his aim is to identify predictive biomarkers to help improve pregnancy outcomes. Ali's motivation stems from his personal experience following the premature birth of his son.

Dr Claus T Christophersen, PhD, MSc, is a molecular microbiologist specialising in the role and impact of the microbiome in health. He participates in multidisciplinary research to understand how to the microbiome interacts with the host and how to manipulate it to can improve health or prevent diseases. He has an MSc from the University of Copenhagen and a PhD from the University of Western Australia. He then undertook a post-doctoral appointment and later became a research scientist and team leader in CSIRO Food and Nutrition before returning to Perth and taking up positions at ECU and Curtin University. He leads the WA Human Microbiome Collaboration Centre (WAHMCC) at Curtin University.

Jeffrey Keelan is Head of the School of Biomedical Sciences and Professor in the Division of Obstetrics and Gynaecology, University of Western Australia. He has 38 years' experience in biomedical research and medical science and has worked and published in the areas of obstetrics, reproductive biology, endocrinology, pharmacology, toxicology, immunology, microbiology and nanomedicine. His current research is focused on the pharmacological treatment of intraamniotic infection/inflammation for the prevention of preterm birth; placental health and dysfunction; nanoparticle-based drug delivery in pregnancy; the intrauterine microbial and endocrine environment; and the role of the microbiome in pregnancy, parturition and early life.

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Targeting host-microbial interactions to develop otitis media therapies

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Abstract. Otitis media (OM; middle ear infection) is the most common reason for pre-school children to visit a doctor, be prescribed antimicrobials, or undergo surgery. Recent Cochrane reviews of clinical trials have identified that antibiotics and grommet surgery are only moderately effective in treating OM, with recurrent or persistent infection observed in one-third of children. Research efforts are focusing on developing improved therapies to treat OM and prevent disease recurrence. The recurrent nature of OM is mostly due to the persistence of bacterial pathogens within established biofilm in the middle ear. Promising novel therapies are harnessing host-microbe interactions to disrupt middle ear biofilm and permit antibiotics to work more effectively. New approaches are also being developed to prevent OM, including new vaccines and mining the host respiratory microbiome to develop novel bacterial therapies. This review describes how our improved knowledge of human and microbial interactions is driving development of OM therapies to improve health outcomes for children in Australia and worldwide.

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Globally, there are ~709 million cases of acute otitis media (OM), ~31 million cases of chronic OM and 21 000 deaths from OM complications each year[1]. In Australia, almost every child will experience an episode of OM by their second birthday. One quarter of Australian children will suffer from recurrent or persistent OM and hearing loss, for which grommet surgery is recommended to improve hearing and reduce the risk of infection. Approximately 35 000 surgeries for OM are conducted each year in Australia[2].

Acute OM (AOM) involves inflammation of the eardrum, which is often painful and associated with fever. Children that suffer from recurrent AOM (three episodes in 6 months or ≥4 in 12 months) are usually referred for grommet surgery (Figure 1*a*). In some children bacteria are never fully cleared and mucous (generated by the child's immune response to presence of bacteria) persists behind the eardrum; this is known as OM with effusion (OME) or 'glue ear'. Figure 1*b* (and Video S1, available as Supplementary material to this paper) shows aspiration of the sticky glue from the middle ear by an ENT surgeon. OME can occur without a preceding AOM episode. Persistent 'glue' in a child's ear results in conductive hearing loss and if left untreated can have a devastating impact on a child's learning, and social and emotional well-being[2,3]. This complication disproportionately affects Aboriginal and Torres Strait Islander children, who suffer the highest reported rates of OM and associated hearing loss in the world – more than double the incidence in non-Aboriginal Australian children[2].

Potential OM pathogens (otopathogens) reside in the nasopharynx and adenoids, usually as asymptomatic colonisers. Transition from colonisation of the upper respiratory tract to middle ear infection often involves a preceding respiratory virus infection, which aids otopathogen ascension to the middle ear through promotion of bacterial proliferation and increased mucous production[4]. Nontypeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and *Moraxella catarrhalis* are the leading otopathogens[5]. We have shown that these otopathogens survive in the 'glue' (biofilm) in the middle ear[6,7] (also shown in Figure 2), where they are up to 1000 times more resistant to antimicrobials and can evade host immune defences[8]. Recently, we have demonstrated that children with bacterial otopathogens detected in their middle ear at the time of grommet surgery are three times more likely to require repeat OM surgery[9]. Thus, ensuring that otopathogens are cleared from the middle ear at the time of first grommet surgery could be a strategy for preventing disease recurrence. Preventing the first episode of OM would be even better.

Current treatment strategies for OM

Antimicrobials

Cochrane reviews of randomised clinical trials (RCTs) have indicated that treating AOM and OME with antimicrobials only has a modest benefit on the symptoms of OM[10]. Long-term low-dose antibiotic treatment has been shown to prevent occurrence of AOM in high-risk children[11], but this must be balanced against the risk of adverse effects such as diarrhoea. Antibiotic use, particularly prolonged low-dose use, can also contribute to the growing threat of antimicrobial resistance.

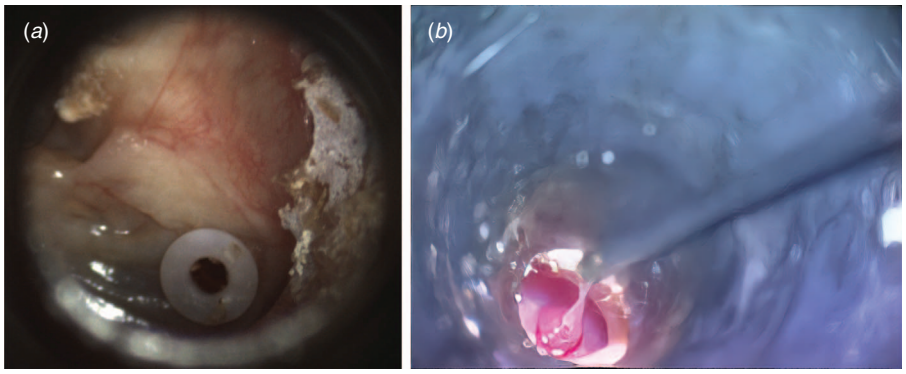

Figure 1. Grommet insertion and removal of effusion from the middle ear. (*a*) Otoscopy image of a grommet (small plastic tube) surgically inserted into the tympanic membrane for treatment of acute otitis media or otitis media with effusion (OME) (image courtesy of Clinical A/Professor Jafri Kuthubutheen). (*b*) Aspiration of middle ear fluid 'glue' from a child with OME, via an incision in the tympanic membrane and prior to grommet insertion (image courtesy of Professor Harvey Coates AO).

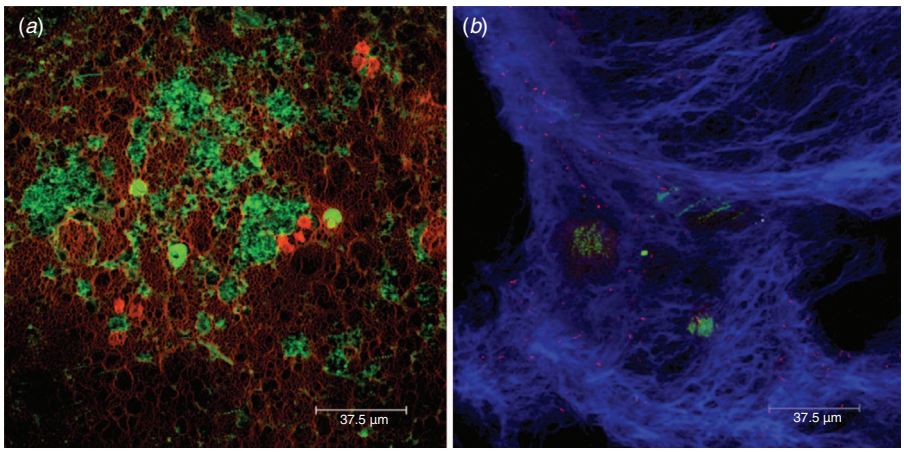

Figure 2. Maximum projection Confocal Laser Scanning microscopy images demonstrating presence of biofilm in middle ear effusion from a child undergoing grommet surgery for recurrent acute otitis media. (*a*) Live/dead staining of middle ear effusion demonstrating presence of live bacteria (green) surrounded by extracellular host DNA (red) within mature bacterial biofilms. (*b*) Fluorescence *in situ* hybridisation on the same middle ear effusion demonstrating multi-species bacterial biofilms using the following 16s rRNA probes: universal bacterial probe (red), *S. pneumoniae* (green), and *H. influenzae* (grey) plus Hoechst 33342 staining for all DNA (blue). *S. pneumoniae* and other unidentified bacteria were observed within biofilms throughout the host DNA in the middle ear fluid. Scale bar = 37.5 µm.

Surgery

Evidence on the effectiveness of OM surgery for OM is poor; a Cochrane review of five RCTs revealed only low-quality evidence for the benefits of grommet surgery[12]. While grommets do improve hearing in the short-term, they can also block with pus or lead to persistent otorrhoea (runny ears)[13]. Furthermore, disease often recurs in children, with >30% of patients returning for repeat grommets[13]. While private surgery waitlists for grommet surgery are only 4 to 6 weeks, the current wait time for grommet surgery at public Australian hospitals is unacceptably long at two years. This is an exceptionally long time for a child to suffer from reduced hearing, which can have major impacts on speech development, education outcomes and social and emotional wellbeing[2,3].

Current clinical preventative strategies for OM

Vaccines

Vaccination remains the gold standard for preventing infections. However, because NTHi has high strain diversity and no polysaccharide capsule, and *S. pneumoniae* has high serotype diversity, it is challenging to develop effective OM vaccines. Currently no OM-targeted vaccines are licensed[14].

Probiotics

Both orally and nasally delivered Lactobacillus and α-Streptococci species can have a moderate untargeted impact on recurrent OM[15]. However, there is no evidence that probiotics protect against initial episodes of OM (Cochrane review of 16 RCTs)[15].

Development of novel therapies to treat OM

Thermoresponsive ototopical gels

Hydrogels that are liquid at room temperature and gel-like at 37°C can be used for controlled delivery of OM therapies. Otiprio® is a new licenced therapy that delivers the antibiotic ciprofloxacin over 10–14 days in a thermoresponsive gel[16]. This gel can be applied into the middle ear at the time of grommet surgery, removing the need for parental application of antibiotic drops and thereby enhancing

compliance. However, antibiotics, even when delivered in a slow-release gel, will have limited effect on established biofilms.

Anti-biofilm treatments

Since the discovery of biofilm in the middle ear of OM patients[17], it is now widely accepted that persistence of infection and recalcitrance to treatment is predominantly driven by biofilm. Biofilms in the ears of children with OM are composed of a combination of host and microbial factors that protect the otopathogens[6]. Researchers are targeting biofilms to enhance treatment for recurrent AOM and chronic OME.

(1) *Therapeutic anti-biofilm vaccine*: antibodies to NTHi proteins (PilA and OmpP5) have been shown to disrupt established NTHi biofilms in the chinchilla model of OM[18]. The biofilm destabilisation is antibody mediated and occurs by targeting the type IV pilus (PilA) responsible for twitching motility, and also the tightly co-regulated quorum signalling molecule (LuxS), both of which are essential for biofilm formation and dispersal[18]. Otopathogens released from the destabilised biofilm are highly susceptible to antibiotics[19]. The PilA protein is included in a trivalent sub-unit NTHi vaccine that was tested in clinical trials in adults with chronic lung disease[20]. While PilA vaccination was safe and induced high antibody titres in the trial, the ability of these antibodies to destabilise OM biofilms in humans has not been assessed. Future trials with antibiofilm therapeutic vaccines in children with chronic NTHi OME are warranted.

(2) *Antibody therapy*: Bacterial extracellular DNA (eDNA) is abundant within bacterial biofilms. This eDNA is arranged in lattices and the critical protein that maintains the biofilm structure is integration host factor (IHF). IgG or Fab-fragments targeting protective epitopes within the DNA-binding tip domains of IHF have been shown to disrupt established biofilms *in vitro* and to mediate resolution of disease in the chinchilla OM NTHi biofilm model[21].

(3) *Anti-neutrophil extracellular trap (NET) therapy*: Dornase alfa (Pulmozyme®) is a DNAse-based therapy routinely used to breakdown biofilm in the lungs of cystic fibrosis (CF) patients. We have shown that Pulmozyme® breaks down NET-derived DNA in middle ear biofilms from OM patients to allow antibiotics to effectively kill the remaining otopathogens *in vitro*[6]. Our Phase I trial (CTN#2011/0635) in 60 children demonstrated that Pulmozyme® application into the middle ear at time of grommet surgery was safe, with no adverse events (manuscript in preparation). Our current Phase II randomised control trial is assessing safety and effectiveness of five daily applications of Pulmozyme® post-surgery (ACTRN12619001306101). Study end-points include safety and tolerability of an extended dosing regimen, recurrence of OM, and need for repeat surgery within 2 years of treatment.

Development of novel therapies to prevent OM

Vaccines

Progress on vaccine development for OM prevention was reviewed following the 2019 international OM meeting[14]. In brief, multi-species vaccines are required to prevent OM but their development is challenging. However, vaccines against NTHi and *M. catarrhalis*, and pneumococcal vaccines with broader serotype coverage, are all in current clinical trials. Vaccines against respiratory viruses are also useful in preventing OM, as demonstrated for influenza[14], and must be tested for new vaccines where possible, i.e. respiratory syncytial virus vaccines. Development of anti-biofilm prophylactic vaccines hold great promise with pre-clinical models demonstrating protection from biofilm formation in the middle ear[22].

Microbiome-derived probiotic therapies

The human respiratory microbiome has been described as 'the gatekeeper to respiratory health'[23] and a potential source of novel therapies. The use of respiratory commensal bacteria as probiotics, rather than gut commensals, for OM prevention is under investigation, with evidence of effectiveness in some but not all studies[24]. We demonstrated that the human respiratory commensal *Haemophilus haemolyticus* can be used to inhibit NTHi colonisation and infection of human respiratory epithelium *in vitro*[25]. In addition, intranasal administration of a closely related murine commensal, *Muribacter muris*, prevented NTHi colonisation and development of NTHi OM in mice[26]. Inflammatory responses to NTHi were curbed in mice receiving *M. muris*[26], which is important given that inflammation plays a major role in OM pathogenesis including neutrophil recruitment and NET formation. We are now undertaking a first-in-human study on the safety and tolerability of intranasally delivered *H. haemolyticus* to healthy adults prior to clinical trials in children to assess impact on OM prevention.

Conclusions

Fundamental research into human-microbial interactions involved in OM has led to significant advances in developing novel approaches to treat and prevent OM. Engaging stakeholder recognition in the value of OM prevention is essential to ensure further investment in development of these new OM therapies. Better treatment and prevention of OM will improve antimicrobial stewardship and conserve healthcare resources, and more importantly help bring equity to hearing health and educational outcomes: when kids can hear, they can learn.

Conflicts of interest

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References

1. Monasta, L. *et al*. (2012) Burden of disease caused by otitis media: systematic review and global estimates. *PLoS One* **7**, e36226. doi:10.1371/journal.pone.0036226
2. Australian Institute of Health and Welfare (AIHW) (2018) Australia's Health 2018.
3. Homøe, P. *et al*. (2020) Panel 5: Impact of otitis media on quality of life and development. *Int. J. Pediatr. Otorhinolaryngol.* **130**(Suppl 1), 109837. doi:10.1016/j.ijporl.2019.109837
4. Massa, H.M. *et al*. (2009) Otitis media: viruses, bacteria, biofilms and vaccines. *Med. J. Aust.* **191**(S9), S44–S49. doi:10.5694/j.1326-5377.2009.tb02926.x
5. Ngo, C.C. *et al*. (2016) Predominant bacteria detected from the middle ear fluid of children experiencing otitis media: a systematic review. *PLoS One* **11**, e0150949. doi:10.1371/journal.pone.0150949
6. Thornton, R.B. *et al*. (2013) Neutrophil extracellular traps and bacterial biofilms in middle ear effusion of children with recurrent acute otitis media–a potential treatment target. *PLoS One* **8**, e53837. doi:10.1371/journal.pone.0053837
7. Thornton, R.B. *et al*. (2011) Multi-species bacterial biofilm and intracellular infection in otitis media. *BMC Pediatr.* **11**, 94. doi:10.1186/1471-2431-11-94
8. Bakaletz, L.O. (2012) Bacterial biofilms in the upper airway – evidence for role in pathology and implications for treatment of otitis media. *Paediatr. Respir. Rev.* **13**, 154–159. doi:10.1016/j.prrv.2012.03.001
9. Seppanen, E.J. *et al*. (2020) Bacterial reservoirs in the middle ear of otitis-prone children are associated with repeat ventilation tube insertion. *Pediatr. Infect. Dis. J.* **39**, 91–96. doi:10.1097/INF.0000000000002541
10. Venekamp, R.P. *et al*. (2016) Antibiotics for otitis media with effusion in children. *Cochrane Database Syst. Rev.* CD009163. doi:10.1002/14651858.CD009163.pub3
11. Leach, A.J. and Morris, P.S. (2006) Antibiotics for the prevention of acute and chronic suppurative otitis media in children. *Cochrane Database Syst. Rev.* CD004401. doi:10.1002/14651858.CD004401.pub2
12. Venekamp, R.P. *et al*. (2018) Grommets (ventilation tubes) for recurrent acute otitis media in children. *Cochrane Database Syst. Rev.* CD012017. doi:10.1002/14651858.CD012017.pub2
13. Schilder, A.G. *et al*. (2017) Panel 7: otitis media: treatment and complications. *Otolaryngol. Head Neck Surg.* **156**(Suppl), S88–S105. doi:10.1177/0194599816633697
14. Alderson, M.R. *et al*. (2020) Panel 8: vaccines and immunology. *Int. J. Pediatr. Otorhinolaryngol.* **130**(Suppl 1), 109839.
15. Scott, A.M. *et al*. (2019) Probiotics for preventing acute otitis media in children. *Cochrane Database Syst. Rev.* CD012941. doi:10.1002/14651858.CD012941.pub2
16. Edmunds, A.L. (2017) Otiprio: an FDA-approved ciprofloxacin suspension gel for pediatric otitis media with effusion. *P&T* **42**, 307–311.
17. Bakaletz, L.O. (2007) Bacterial biofilms in otitis media: evidence and relevance. *Pediatr. Infect. Dis. J.* **26**(Suppl), S17–S19. doi:10.1097/INF.0b013e318154b273
18. Novotny, L.A. *et al*. (2015) Antibodies against the majority subunit of type IV pili disperse nontypeable *Haemophilus influenzae* biofilms in a LuxS-dependent manner and confer therapeutic resolution of experimental otitis media. *Mol. Microbiol.* **96**, 276–292. doi:10.1111/mmi.12934
19. Mokrzan, E.M. *et al*. (2020) Nontypeable *Haemophilus influenzae* newly released (NRel) from biofilms by antibody-mediated dispersal versus antibody-mediated disruption are phenotypically distinct. *Biofilm.* **2**, 100039. doi:10.1016/j.bioflm.2020.100039
20. Wilkinson, T.M.A. *et al*. (2019) Non-typeable *Haemophilus influenzae* protein vaccine in adults with COPD: a phase 2 clinical trial. *Vaccine* **37**, 6102–6111. doi:10.1016/j.vaccine.2019.07.100
21. Novotny, L.A. *et al*. (2021) Humanized anti-DNABII fab fragments plus ofloxacin eradicated biofilms in experimental otitis media. *Laryngoscope.* doi:10.1002/lary.29497
22. Novotny, L.A. *et al*. (2017) Transcutaneous immunization with a band-aid prevents experimental otitis media in a polymicrobial model. *Clin. Vaccine Immunol.* **24**, e00563-16. doi:10.1128/CVI.00563-16
23. Man, W.H. *et al*. (2017) The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat. Rev. Microbiol.* **15**, 259–270. doi:10.1038/nrmicro.2017.14
24. Marsh, R.L. *et al*. (2020) Panel 4: Recent advances in understanding the natural history of the otitis media microbiome and its response to environmental pressures. *Int. J. Pediatr. Otorhinolaryngol.* **130**(Suppl 1), 109836. doi:10.1016/j.ijporl.2019.109836
25. Pickering, J.L. *et al*. (2016) *Haemophilus haemolyticus* interaction with host cells is different to nontypeable *Haemophilus influenzae* and prevents NTHi association with epithelial cells. *Front. Cell. Infect. Microbiol.* **6**, 50. doi:10.3389/fcimb.2016.00050
26. Granland, C.M. *et al*. (2020) Nasal delivery of a commensal pasteurellaceae species inhibits nontypeable *Haemophilus influenzae* colonization and delays onset of otitis media in mice. *Infect. Immun.* **88**, e00685-19. doi:10.1128/IAI.00685-19

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Neisseria species and their complicated relationships with human health

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Abstract. *Neisseria* spp. are a transient low abundance member of the human microbiome. This species contains the very well described pathogens, *Neisseria gonorrhoeae* and *N. meningitidis*. Recent advances in molecular typing have revealed that this genus is more diverse than previously thought and that commensal species may have important roles in inhibiting the growth the pathogens. This short review summates these new findings and examines the evidence that the relatively under-reported *Neisseria* commensal species maybe beneficial to human health.

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In 1879 Albert Ludwig Neisser observed diplococci found within neutrophils present in urethral exudates of men and women suffering from gonorrhoea and gonorrhoeal conjunctivitis. This organism was later named *Neisseria gonorrhoeae* and marks the first ever description of a member of the genus *Neisseria*[1]. The genus *Neisseria* belongs to the family *Neisseriaceae* within the phylum β-*Proteobacteria*[2]. Other genera of the family *Neisseriaceae* of medical importance include *Kingella* and *Eikinella*[2].

The *Neisseria* genus is larger and more diverse than first thought

The *Neisseria* genus contains diverse species inhabiting mammals, reptiles and environmental sites[3]. Members of the genus are Gram-negative, generally diplococci. Some *Neisseria* species such as *N. weaveri*, *N. elongata* and *N. bacilliformis* do not conform to the general diplococcus morphology, instead existing as chains of bacilli or filaments[4]. Other classical characteristics of the genus *Neisseria* include lack of motility, absence of flagella, aerobic fermentation of sugars and oxidase production. *Neisseria* speciation is continuously being revised and so far there are 10 established species associated with humans (Table 1) with a further seven recently identified from a nasopharyngeal carriage study in an African population[5]. The current robust phylogeny of this species has been developed by applying multi-locus sequence typing (MLST)[6,7]. The MLST scheme uses the single nucleotide polymorphisms in each gene to create a unique sequence type (ST) for every isolate. STs can be grouped into larger clusters based on their similarity to one another. The schemes use different numbers of genes with the basic approach using seven housekeeping genes, ribosomal MLST (rMLST) using 53 ribosomal genes[8] and a core genome MLST (cgMLST) using 246 conserved loci[9]. This has resulted in the condensation of older isolates classified as *N. subflava* biovar *subflava*, *perflava*, *flava* and *flavescens* into a single species, *N. subflava*[9]. Isolates previously termed *N. sicca* are now variants of *N. mucosa*[9] and those previously termed *N. mucosa* var *heidelbergenisis* are now called *N. oralis*[10]. Genomic approaches have been more robust than matrix-assisted laser desorption ionisation-time of flight mass spectroscopy (MALDI-ToF) at discriminating these species due to their close relatedness[11]. In the case of laboratory diagnostic identification, whole genome sequencing is the best approach to identify an unknown *Neisseria* sp.

Neisseria spp. that act as pathogens in the human host

Neisseria spp. have multiple modes of interfacing with the human host. *N. gonorrhoeae* is considered to be a true pathogen[12] as it elicits an inflammatory response upon urethral infection of the human male and causes a delayed inflammatory response, pelvic inflammatory disease, in women. Interestingly, although classified as a pathogen it can asymptomatically colonise the oral mucosa and anorectal sites that self-resolve over 4–12 months[13]. *N. meningitidis*, the causative agent of invasive meningococcal disease (IMD), is considered an opportunistic pathogen. Whereas *N. gonorrhoeae* is highly clonal[7], *N. meningitidis* has diversified into at least 11 clonal complexes that are highly associated with the risk of IMD[14]. A much wider array of genetic lineages are colonisers of the human host but act as commensals as they are infrequently associated with IMD. These two groups are broadly distinguished by the possession of a capsule polysaccharide synthesis (*cps*) operon. Among many virulence factors[15], the possession of a capsule by *N. meningitidis* is

Table 1. Summary of characteristics of human commensal *Neisseria* species.

Neisseria spp.	Micro/macroscopic morphology	Host	Biotic relationship	Site/niche	Reference
N. meningitidis	Gram-negative diplococcus	Human	Commensal and/or pathogen	Nasopharynx (commensal/pathogen)	19
				Urethra	
N. gonorrhoeae	Gram-negative diplococcus	Human	Pathogen	Mucous membranes of nasopharynx, genital mucosa, urethra, conjunctiva, rectum	13
N. bacilliformis	Gram-negative bacilli or filamentous rods	Human (may not be human exclusive)	Commensal	Mucous membranes of oral cavity	8,9
N. lactamica	Gram-negative diplococcus	Human	Commensal	Nasopharynx	9,39
	Yellow pigment production, some strains haemolytic on horse blood agar				
N. mucosa	Gram-negative diplococcus	Human	Commensal	Nasopharynx, dental plaque and buccal mucosa	9
	Most strains non-pigmented, some produce grey to yellow pigment (formerly known as *N. sicca*)				
N. cinerea	Gram-negative diplococcus	Human	Commensal	Respiratory tract: nasopharynx, sputum	9,40
	Some strains produce yellow pigment in colonies			Urogenital tract: vagina, cervix, urethra and urine	
				Other sites: eyes, ears, blood	
N. elongata	Gram-negative filamentous rods	Human	Commensal	Nasopharynx, blood	9,24
N. oralis	Gram-negative diplococcus, (may be present in chains, formerly known as *N. mucosa* var heidelbergensis)	Human	Commensal	Nasopharynx, blood	10
				Gingival plaque	
N. polysaccharea	Gram-negative diplococcus	Human	Commensal	Nasopharynx	9,41
N. subflava	Gram-negative diplococcus	Human	Commensal	Gingival crevice/upper respiratory tract	9
	Yellow colonies				
	Spontaneous agglutination in saline (formerly known as *N. subflava biovar subflava*, *N. perflava*, *N. flava*, *N. flavescens*)				

a key factor enabling survival of IMD-causing bacteria within the blood stream to cause bacteraemia and meningitis. This feature is the basis of genogrouping isolates by quantitative real-time PCR in meningococcal carriage studies. Isolates that are non-disease-causing and disease-causing isolates are stratified by the presence of a capsule null locus (*cnl*) and capsule transporter A (*ctrA*), respectively[16]. Meningococcal carriage studies have shown that the prevalence of nasopharyngeal carriage of the meningococcus ranges from 10–30% dependent upon a variety of community and behavioural factors[14]. However, since the incidence of IMD is much lower than this, other factors are involved in the risk of progressing to IMD after colonisation. This fulcrum rests on the virulence of the isolate and the underlying health of the host[17,18]. Until recently, *N. meningitidis* was not associated with urogenital disease and was considered to be a transient asymptomatic coloniser of the urogenital compartment. This concept was dramatically revised with the report in 2017 of an outbreak of urogenital urethritis attributed to meningococci closely related to an IMD outbreak clade[19]. A retrospective review of published case reports of meningococcal disease has uncovered consistent reporting of sporadic cases of horizontal mother to child transmission in pregnancy resulting in rare cases of sepsis, anorectal infection and conjunctivitis[20].

Neisseria spp. that are low abundance, transient commensals of the human host

In comparison to the two pathogenic species, the remaining eight species are atypical infectious disease agents[3, 21]. Collectively they

are sporadically associated with a wide variety of conditions usually in immunocompromised patients[21]. Since they are not widely known as infectious disease agents, it is also possible that the reports of their involvement in these disease manifestations is under-reported. Nevertheless, genomic comparisons of these commensal species with the pathogenic *N. meningitidis* shows that they lack multiple virulence determinants[22] supporting the conclusion that they are naturally commensal and act as opportunistic pathogens in a disregulated host immune environment. Prevalence studies have typically examined pharyngeal carriage and have shown that all of these species are transient low abundance (<2% abundance) members of the human microbiome. *N. lactamica* has the highest prevalence of all species and with the highest incidence in children under the age of 4 (14%) before declining in young adults[23]. *N. polysaccharea* also showed a similar distribution as *N. lactamica* but at a much lower incidence of 2%. In this study *N. bergeri* and *N. subflava* had very low prevalence and showed no age-related variation in incidence. Co-colonisation studies have not been performed recently, but an older study from the 1980s that used culture as the means of detection, found multiple *Neisseria* spp. occurred in 57% of people while 41% of carriage was with *N. subflava* alone[24]. The high prevalence of *N. subflava* appears to be due to its role as a contributor to periodontal disease. Although multiple *Neisseria* spp. are present in both healthy teeth and dental caries samples, an increase in the abundance of *N. subflava* is a key signal as the microbial community changes in composition to become acid-secreting, resulting in tooth enamel erosion[25].

The role of *Neisseria* spp. in the human microbiome

Human microbiome studies have begun to unravel some relationships of the *Neisseria* spp. within their relevant mucosal microbiome communities. Unfortunately, *Neisseria* spp. are typically reported at the genus level as variation in the 16S rRNA alone is insufficient to speciate them. Nevertheless, some generalities can be gained from the current literature. Numerous studies have shown that *Neisseria* spp. are absent from normal flora in the vulvovaginal mucosal surfaces of women[26]. This suggests that the isolation of any *Neisseria* spp. from this compartment should be investigated as a potential pathogen related to an infection particularly urethritis[3,21]. Commensal *Neisseria* spp. are transient, low abundance residents of the rhinopharynx and oropharynx[27] that are not associated with any known disease-state[28].

There are hints that there are complex interference patterns at both intra- and inter-species levels that influence colonisation by *Neisseria* spp. Many of these interactions have been examined through the lens of preventing or interfering with colonisation by the pathogens. Exposure to *N. gonorrhoeae* does not necessarily result in human infection. In surveys of human disease, the risk of contracting gonorrhoea has been linked to a syndrome termed bacterial vaginosis, in which the microbiome has a reduced abundance of *Lactobacillus* sp.[29]. Although co-culture of the two species confirms *Lactobacillus* sp. will inhibit *N. gonorrhoeae* growth, probiotic treatment of mice with *Lactobacillus* shows no efficacy in mouse models of gonorrhoea infection[30]. *Streptococcus pneumoniae* has been shown to inhibit *N. meningitidis* using two mechanisms: the secretion of hydrogen peroxide[31] and a neuraminidase[32]. Inter-species antagonism is also a feature of the commensal *Neisseria* spp. against both *N. gonorrhoeae* and *N. meningitidis*. *N. cinerea* and *N. lactamica* impair early colonisation steps and reduce meningococcal invasion into host cells[33,34] while *N. mucosa* secretes a small molecule secondary metabolite that inhibits *N. gonorrhoeae*[35]. However, all commensal *Neisseria* spp. could kill *N. gonorrhoeae* through a DNA-dependent mechanism[36]. This mechanism is dependent on the expression of type IV pili, which enable the uptake of DNA into the bacterial cell. The DNA from the commensal bacteria have a different methylation pattern and this appears to poison the gonococcal and meningococcal bacteria[33]. Direct synergism between *Neisseria* spp. and other species has not been extensively reported. However, a recent innovative model of meningococcal colonisation conducted by Audry *et al*.[37] showed that meningococcal colonisation of the human oropharyngeal site may not elicit an immediate inflammatory response as the bacteria can be trapped in the mucus layer, preventing invasion of the mucosal epithelium. This state of homeostasis can be perturbed by co-colonisation with other bacteria, and in this model, *Streptococcus mitis* but not *Moraxella catarrhalis* triggered the escape of the meningococcus from the mucus layer and invasion into the host cells. *S. mitis* potentiated growth of the meningococcus by degrading the mucins.

Future directions

In summary, the taxonomy of the genus *Neisseria* is continually being redefined by modern molecular typing tools and the recent observation that the diversity of this group remains largely unexplored. This genus contains species that are either pathogenic or commensal with humans, whereas *N. meningitidis* contains clonal complexes that are pathogenic or commensal. Since its discovery 142 years ago, the interest in this genus has been driven by the medical interest in devising preventative measures against gonorrhoea and meningitis. Other members of this genus, such as *N. lactamica* have been investigated as a probiotic intervention strategy against IMD[34], while the recent observation that commensal

Neisseria spp. may kill *N. gonorrhoeae* via a DNA-dependent mechanism has been recently patented (International Patent Application No. PCT/US2015/048114). Future work is likely to focus on whether commensal *Neisseria* spp. have a benefit to human health and are necessary for development of a healthy immune system.

Conflicts of interest

The author declares no conflicts of interest.

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References

1. Neisser, A. (1897) Uber eine der Gonorrhoe eigenthumliche Micrococcusform. *Centralblatt fur medizinische Wissenschaft* **17**, 497–500.
2. Bennett, J.S. *et al*. (2014) The genus *Neisseria*. In *The Prokaryotes – Alphaproteobacteria and Betaproteobacteria*, Rosenberg, E. *et al*. (eds.). pp. 881–900. Springer-Verlag, Berlin, Heidelberg.
3. Liu, G. *et al*. (2015) Non-pathogenic *Neisseria*: members of an abundant, multihabitat, diverse genus. *Microbiology (Reading)* **161**, 1297–1312. doi:10.1099/mic.0.000086
4. Bøvre, K. (1980) Progress in classification and identification of Neisseriaceae based on genetic affinity. In *Microbiological Classification and Identification*. pp. 55–72. Academic Press Inc., London.
5. Diallo, K. *et al*. (2019) Genomic characterization of novel *Neisseria* species. *Sci. Rep.* **9**, 13742. doi:10.1038/s41598-019-50203-2
6. Maiden, M.C. *et al*. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. USA* **95**, 3140–3145. doi:10.1073/pnas.95.6.3140
7. Bennett, J.S. *et al*. (2007) Species status of *Neisseria gonorrhoeae*: evolutionary and epidemiological inferences from multilocus sequence typing. *BMC Biol.* **5**, 35. doi:10.1186/1741-7007-5-35
8. Jolley, K.A. *et al*. (2012) Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. *Microbiology (Reading)* **158**, 1005–1015. doi:10.1099/mic.0.055459-0
9. Bennett, J.S. *et al*. (2012) A genomic approach to bacterial taxonomy: an examination and proposed reclassification of species within the genus *Neisseria*. *Microbiology (Reading)* **158**, 1570–1580. doi:10.1099/mic.0.056077-0
10. Bennett, J.S. *et al*. (2013) Genome sequence analyses show that *Neisseria oralis* is the same species as '*Neisseria mucosa* var. *heidelbergensis*'. *Int. J. Syst. Evol. Microbiol.* **63**, 3920–3926. doi:10.1099/ijs.0.052431-0
11. Cunningham, S.A. *et al*. (2014) Misidentification of *Neisseria polysaccharea* as *Neisseria meningitidis* with the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* **52**, 2270–2271. doi:10.1128/JCM.00664-14
12. Casadevall, A. and Pirofski, L.A. (2000) Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect. Immun.* **68**, 6511–6518. doi:10.1128/IAI.68.12.6511-6518.2000
13. Fairley, C.K. *et al*. (2019) Models of gonorrhoea transmission from the mouth and saliva. *Lancet Infect. Dis.* **19**, e360–e366. doi:10.1016/S1473-3099(19)30304-4
14. Acevedo, R. *et al*. (2019) The Global Meningococcal Initiative meeting on prevention of meningococcal disease worldwide: epidemiology, surveillance, hypervirulent strains, antibiotic resistance and high-risk populations. *Expert Rev. Vaccines* **18**, 15–30. doi:10.1080/14760584.2019.1557520
15. Mullally, C.A. *et al*. (2021) Evolutionary pathways for commensalism and hypervirulence in *N. meningitidis*. *Microbial Genomics* [In press].
16. Wang, X. *et al*. (2012) Clinical validation of multiplex real-time PCR assays for detection of bacterial meningitis pathogens. *J. Clin. Microbiol.* **50**, 702–708. doi:10.1128/JCM.06087-11
17. Davila, S. *et al*. (2010) Genome-wide association study identifies variants in the CFH region associated with host susceptibility to meningococcal disease. *Nat. Genet.* **42**, 772–776. doi:10.1038/ng.640
18. Gianchecchi, E. *et al*. (2016) *N. meningitidis* and TLR polymorphisms: a fascinating immunomodulatory network. *Vaccines (Basel)* **4**, 20. doi:10.3390/vaccines4020020
19. Tzeng, Y.L. *et al*. (2017) Emergence of a new *Neisseria meningitidis* clonal complex 11 lineage 11.2 clade as an effective urogenital pathogen. *Proc. Natl. Acad. Sci. USA* **114**, 4237–4242. doi:10.1073/pnas.1620971114
20. Ladhani, S.N. *et al*. (2020) Meningococcal disease and sexual transmission: urogenital and anorectal infections and invasive disease due to *Neisseria meningitidis*. *Lancet* **395**, 1865–1877. doi:10.1016/S0140-6736(20)30913-2
21. Humbert, M.V. and Christodoulides, M. (2019) Atypical, yet not infrequent, infections with *Neisseria* Species. *Pathogens* **9**, 10. doi:10.3390/pathogens9010010
22. Marri, P.R. *et al*. (2010) Genome sequencing reveals widespread virulence gene exchange among human *Neisseria* species. *PLoS One* **5**, e11835. doi:10.1371/journal.pone.0011835
23. Diallo, K. *et al*. (2016) Pharyngeal carriage of *Neisseria* species in the African meningitis belt. *J. Infect.* **72**, 667–677. doi:10.1016/j.jinf.2016.03.010
24. Knapp, J.S. (1988) Historical perspectives and identification of *Neisseria* and related species. *Clin. Microbiol. Rev.* **1**, 415–431. doi:10.1128/CMR.1.4.415
25. Peterson, S.N. *et al*. (2013) The dental plaque microbiome in health and disease. *PLoS One* **8**, e58487. doi:10.1371/journal.pone.0058487
26. Kalia, N. *et al*. (2020) Microbiota in vaginal health and pathogenesis of recurrent vulvovaginal infections: a critical review. *Ann. Clin. Microbiol. Antimicrob.* **19**, 5. doi:10.1186/s12941-020-0347-4
27. Santacroce, L. *et al*. (2020) The human respiratory system and its microbiome at a glimpse. *Biology (Basel)* **9**, 318. doi:10.3390/biology9100318
28. Bogaert, D. *et al*. (2011) Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One* **6**, e17035. doi:10.1371/journal.pone.0017035
29. Bautista, C.T. *et al*. (2016) Bacterial vaginosis: a synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. *Mil. Med. Res.* **3**, 4. doi:10.1186/s40779-016-0074-5
30. Jerse, A.E. *et al*. (2011) Estradiol-treated female mice as surrogate hosts for *Neisseria gonorrhoeae* genital tract infections. *Front. Microbiol.* **2**, 107. doi:10.3389/fmicb.2011.00107
31. Pericone, C.D. *et al*. (2000) Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infect. Immun.* **68**, 3990–3997. doi:10.1128/IAI.68.7.3990-3997.2000
32. Shakhnovich, E.A. *et al*. (2002) Neuraminidase expressed by *Streptococcus pneumoniae* desialylates the lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*: a paradigm for interbacterial competition among pathogens of the human respiratory tract. *Infect. Immun.* **70**, 7161–7164. doi:10.1128/IAI.70.12.7161-7164.2002
33. Custodio, R. *et al*. (2020) Commensal *Neisseria cinerea* impairs *Neisseria meningitidis* microcolony development and reduces pathogen colonisation of epithelial cells. *PLoS Pathog.* **16**, e1008372. doi:10.1371/journal.ppat.1008372
34. Li, Y. *et al*. (2006) Immunization with live *Neisseria lactamica* protects mice against meningococcal challenge and can elicit serum bactericidal antibodies. *Infect. Immun.* **74**, 6348–6355. doi:10.1128/IAI.01062-06
35. Aho, E.L. *et al*. (2020) The human microbiome as a focus of antibiotic discovery: *Neisseria mucosa* displays activity against *Neisseria gonorrhoeae*. *Front. Microbiol.* **11**, 577762. doi:10.3389/fmicb.2020.577762
36. Kim, W.J. *et al*. (2019) Commensal *Neisseria* kill *Neisseria gonorrhoeae* through a DNA-dependent mechanism. *Cell Host Microbe* **26**, 228–239 e8. doi:10.1016/j.chom.2019.07.003
37. Audry, M. *et al*. (2019) Airway mucus restricts *Neisseria meningitidis* away from nasopharyngeal epithelial cells and protects the mucosa from inflammation. *MSphere* **4**, e00494-19. doi:10.1128/mSphere.00494-19

38. Han, X.Y. *et al*. (2006) *Neisseria bacilliformis* sp. nov. isolated from human infections. *J. Clin. Microbiol.* **44**, 474–479. doi:10.1128/JCM.44.2.474-479.2006

39. Hollis, D.G. *et al*. (1969) *Neisseria lactamicus* sp. n., a lactose-fermenting species resembling *Neisseria meningitidis*. *Appl. Microbiol.* **17**, 71–77. doi:10.1128/AM.17.1.71-77.1969

40. Knapp, J.S. *et al*. (1984) Characterization of *Neisseria cinerea*, a nonpathogenic species isolated on Martin-Lewis medium selective for pathogenic *Neisseria* spp. *J. Clin. Microbiol.* **19**, 63–67. doi:10.1128/JCM.19.1.63-67.1984

41. Riou, J.Y. *et al*. (1983) A new taxon in the genus *Neisseria*. *Ann. Microbiol. (Paris)* **134**(Suppl B), 257–267. doi:10.1016/S0769-2609(83)80038-6

Biography

Associate Professor Charlene Kahler is a teaching/research academic specialising in bacterial pathogenesis. She obtained her BSc (honours in Microbiology) from the University of Queensland. She completed her PhD in the field of microbiology with Dr John Pemberton at the University of Queensland. She travelled to the USA to undertake post-doctoral training with Dr David Stephens at Emory University (Atlanta, Georgia). In this position, she studied the pathogenesis of *Neisseria meningitidis* and described the biosynthesis pathway of lipooligosaccharide. She returned to Australia to work with Professor John Davies at Monash University where she studied regulatory pathways in *N. gonorrhoeae*. She moved to the University of Western Australia to establish her own laboratory studying both pathogens. She is currently Head of Discipline for Microbiology and Immunology and the Deputy Director of the Marshall Centre for Infectious Diseases Research and Training at University of Western Australia. Her greatest accomplishment is assisting her students through their PhDs and seeing them fulfil their dreams in microbiology. She is thankful to the members of the Centre who contributed to this issue: Dr Tim Inglis, Dr Allison Imrie, Professor Jeff Keelan and Professor Barry Marshall.

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HLA and immunodominance in viral infection: T-cell responses in protection and immunopathogenesis

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Abstract. The protective role of T cells in viral infection is well described. T cells generally mediate anti-viral immune responses via direct cytotoxicity and production of pro-inflammatory cytokines, by providing help to B cells and by promotion of memory responses. A fundamental step in T cell responses involves presentation of viral peptide antigens in the context of human leucocyte antigens (HLA), to the T-cell receptor. HLA are highly polymorphic cell surface molecules that present a vast array of peptides to T cells and induce their activation, differentiation and proliferation into effector cells which can eliminate microbial infection.

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The human HLA molecules were first identified in the early 20th century as transplantation antigens and characterised in the 1950s as transfusion antigens. In 1974 Rolf Zinkernagel and Peter Doherty[1] described the phenomenon of MHC restriction – that killing of virus-infected cells by mouse cytotoxic T cells depended on a combination of viral antigen and mouse H-2 (murine HLA) antigen. Subsequent work over the next decades showed that the human MHC (Major Histocompatibility Complex) encodes the classical MHC Class I and Class II HLA molecules, which present peptide antigens to $CD8^+$ and $CD4^+$ T cells, respectively. The Class I MHC encode the HLA-A, HLA-B, and HLA-C molecules involved in peptide antigen presentation to $CD8^+$ T cells, and the Class II genes encode the HLA-DP, HLA-DQ, HLA-DR molecules that present peptide antigens to $CD4^+$ T cells. Each of the MHC genes is highly polymorphic, encoding a large number of variants differing by up to 20 amino acids and which are capable of binding different peptides; differences within these variants are largely within the peptide-binding sites that make direct contact with the T-cell receptor. More than 20 000 different Class I and Class II alleles have been identified so far[2].

HLA Class I molecules are expressed on the surface of almost all nucleated cells. Class II molecules are constitutively expressed on immune cells including professional antigen presenting cells (dendritic cells, macrophages and monocytes), B cells and activated T cells, and expression can be induced on most cells by interferon-gamma. T cells recognise peptide antigens bound to HLA molecules, as peptide/MHC (pMHC) complexes, via their T-cell receptors. Activated naïve T cells undergo clonal expansion to effector cells that mediate protective immune responses including cytokine secretion and cytotoxicity, and a small percentage of effector cells become long lived memory cells which can be reactivated to mediate protective immunity when the host is challenged months or years later. As all nucleated cells can express class I MHC, activated CTL can kill any infected cell in any tissue and significantly reduce the reservoirs of infection. CTL are critical for control of many acute viral infections and provide protection against secondary infections.

After entering a cell, viruses initiate translation of their proteins. Proteins within the cytosol enter the proteosome where they are cleaved to peptides 8–12 amino acids long. These peptides are transported into the endoplasmic reticulum where they associate with newly synthesised MHC Class I molecules, and the pMHC complex is transferred to the surface of the infected cell where it can interact with the T-cell receptor of $CD8^+$ T cells. Of the thousands of peptides encoded by a virus that can be presented to T cells, in association with a given MHC Class I allele, only a small number of immunodominant peptide antigens induce a response. Class I polymorphism is thought to have evolved as a protective function of the immune response to the large array of microbes we encounter, including emergent and re-emergent viruses, and to protect against pathogen immune evasion. However, both protective and dysfunctional HLA-associated T-cell responses have been described.

Escape from CTL-mediated immune control was first described for HIV-1 in 1991[3]. HLA-B*27-positive HIV-infected individuals are among the elite controllers, antiretroviral-naïve subjects with undetectable viral loads. The HLA-B*27-restricted response to the immunodominant HIV GAG 263-272 KK10 epitope was shown to be associated with slow progression to disease. Mutation within this epitope that prevented peptide attachment to the binding cleft of the Class I molecule abrogated immunogenicity for any HLA-B*27-

positive recipient of the variant virus. Escape mutants were associated with progression to AIDS[4]. Following recognition of the role of HLA in control of HIV replicative capacity, other Class I molecules were linked to protective responses. HLA-mediated control of HIV has been well described within the sub-Saharan African population, in the context of HLA-B*57 and HLA-B*58-restricted HIV p24 Gag-specific responses. The immunodominant HLA-B*57:03-restricted GAG 162-172 KF11 epitope is targeted by the majority of infected individuals expressing HLA-B*57:03. Escape within KF11 is similar to that described for HLA-B*27-KK10 where mutation is at the residue that anchors the peptide to the HLA binding cleft, and can lead to loss of recognition of the epitope by $CD8^+$ T cells if compensatory mutations do not restore viral fitness. These CTL responses directed against the more conserved HIV Gag protein are more effective in long term suppression of viremia than responses against less conserved viral proteins, including HIV Env, which do not have a significant impact on HIV replication capacity and are not associated with control of viremia[5,6]. Genome-wide association studies identified the HLA-peptide binding region as the major factor modulating control of HIV-1 replication in elite controllers[7], supporting the findings of *in vitro* analyses of T-cell function; however, subsequent assessment of HLA-B*57- and HLA-B*27-restricted CD8 T cells showed that elite controllers were differentiated from progressors who expressed the same alleles, on the basis of potency and cross-reactivity of T-cell receptor recognition of HIV-1. The protective effect of HLA alleles is therefore modulated by host TCR usage, which determines viral replication capacity and evolution of immune escape variants[8].

In dengue virus (DENV) infection virus-specific T-cell responses have been shown to be both protective and pathologic. Primary DENV infection induces long lasting immunity[9] to the same DENV serotype but does not provide long term protection against infection with the other three serotypes and people who live in dengue endemic areas will likely be infected multiple times over their lifetimes. Pre-existing cross-reactive memory T cells may be preferentially reactivated to mount an ineffective anti-viral response which does not control viral replication; higher viremia is associated with increased likelihood of developing severe dengue[10]. This phenomenon of original antigenic sin in dengue-specific T-cell responses was described in a Thai population, with HLA-A*11 found in 30% of the southeast Asian population, presenting DENV NS3 130-144 GTS epitope[11]. $CD8^+$ memory T cells in acute phase DENV infection preferentially bound tetramers constructed with serotype-specific GTS epitope peptide variants representing possible earlier dengue infections, and there was an association between magnitude of the T-cell response and disease severity. Skewed memory T-cell responses have been described in other populations: $CD8^+$ T-cell clones specific for an immunodominant HLA-B*55-restricted NS5 329-337 KP9 epitope demonstrated greater functional avidity for variant DENV-2 epitope peptides, in Pacific Islanders recently infected with DENV-1 and who had encountered DENV-2 in a previous epidemic[12].

Polymorphism, particularly in the HLA-A gene, was shown to be associated with increased susceptibility to dengue haemorrhagic fever/severe dengue in a Vietnamese population[13], where HLA-A*11 and HLA-A*24 were considered susceptible genotypes that present epitopes, in the relatively conserved DENV NS3 and NS5, that are both serotype-specific and cross-reactive. Such potentially serotype cross-reactive $CD8^+$ T cells are postulated to contribute to dengue immunopathogenesis in endemic settings that experience regular epidemic transmission of variant DENV. Another study in more than 600 Vietnamese children with severe dengue found the same association with HLA-A*24, where the A*2402/03/10 allele with altered structure in the peptide binding pocket was expressed at higher frequency in children with severe dengue compared to population background groups[14].

Other population-based studies have shown strong protective effects of DENV-specific $CD8^+$ T cells, with repeated DENV exposure in Sri Lankan blood donors driving responses towards CTL recognition of relatively conserved non-structural proteins NS3, NS4B and NS5[15]. HLA-B-restricted responses (B*0702, B*3501, B*4001) were of significantly higher magnitude and greater breadth, and were associated with multifunctional T-cell responses with hierarchy IFN-gamma>TNF-alpha>IL-2, compared with HLA-A responses that were of lower breadth and magnitude. These findings were extended in a Nicaraguan population, where despite differences in DENV variants and epidemiology there was also a strong correlation between HLA type and breadth and magnitude of T-cell responses, including immunodominant responses restricted by HLA-B*3501, an allele that was also associated with protection in the Sri Lankan population. As was described in Vietnam, HLA-A*2402 was subdominant and associated with increased susceptibility to severe disease. Interestingly, B*3501-restricted T cells but not HLA-A*2402-restricted T cells expressed PD-1, and in contrast to other viral infections these PD-1+ $CD8^+$ T cells were associated with activation, not exhaustion, and were proliferative and functional. PD-1 may be a marker of activated and highly functional $CD8^+$ memory T cells in DENV infection[16].

An exhausted $CD8^+$ T-cell phenotype has been described in patients with severe COVID-19[17] but not in patients with more mild disease, suggesting that cellular immune responses are protective. $CD8^+$ and $CD4^+$ T-cell epitopes are being mapped and their immunodominance assessed for common and less frequent HLA alleles, across different population groups. Of great interest is the

observed cross-reactivity in SARS-CoV-2 T-cell responses in healthy unexposed people sampled prior to the pandemic[18], raising the issue of whether pre-existing SARS-CoV-2-reactive memory T cells, likely induced in previous human seasonal coronavirus infection, mediate protection or contribute to immunopathogenesis of COVID-19. These data, in association with a greater understanding of SARS-CoV-2-specific T-cell phenotype and function, will advance our understanding of the correlates of protection and immunopathogenesis and importantly, enhance our understanding of how to best optimise COVID-19 vaccine design.

Conflicts of interest

The authors declare no conflicts of interest.

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References

1. Zinkernagel, R.M. and Doherty, P.C. (1974) Restriction of in vitro T cell-mediated cytoxicity in lymphocytic choriomeningitis within a sungeneic or semiallogeneic system. *Nature* **248**, 701–702. doi:10.1038/248701a0
2. Robinson, J. *et al*. (2020) PD-IMGT/HLA Database. *Nucleic Acids Res.* **48**, D948–D955. doi:10.1093/nar/gkz950
3. Phillips, R.E. *et al*. (1991) Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **354**, 453–459. doi:10.1038/354453a0
4. Goulder, P.J. *et al*. (1997) Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat. Med.* **3**, 212–217. doi:10.1038/nm0297-212
5. Troyer, R.M. *et al*. (2009) Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLoS Pathog.* **5**, e1000365. doi:10.1371/journal.ppat.1000365
6. Kiepiela, P. *et al*. (2007) CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat. Med.* **13**, 46–53. doi:10.1038/nm1520
7. Pereyra, F. *et al*. (2010) The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science* **330**, 1551–1557. doi:10.1126/science.1195271
8. Chen, H. (2012) TCR clonotypes modulate the protective effects of HLA. *Nat. Immunol.* **13**, 691–700. doi:10.1038/ni.2342
9. Imrie, A. *et al*. (2007) Antibody to dengue 1 detected more than 60 years after infection. *Viral Immunol.* **20**, 672–675. doi:10.1089/vim.2007.0050
10. Vaughn, D.W. *et al*. (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J. Infect. Dis.* **181**, 2–9. doi:10.1086/315215
11. Mongkolsapaya, J. *et al*. (2003) Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med.* **9**, 921–927. doi:10.1038/nm887
12. Imrie, A. *et al*. (2007) Differential functional avidity of dengue virus-specific T cell clones for variant peptides representing heterologous and previously encountered serotypes. *J. Virol.* **81**, 10081–10091. doi:10.1128/JVI.00330-07
13. Loke, H. *et al*. (2001) Strong HLA class I-restricted T cell responses in dengue hemorrhagic fever: a double-edged sword? *J. Infect. Dis.* **184**, 1369–1373. doi:10.1086/324320
14. Lan, N.T.P. *et al*. (2008) Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. *PLoS Negl. Trop. Dis.* **2**, e304. doi:10.1371/journal.pntd.0000304
15. Weiskopf, D. *et al*. (2013) Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for $CD8^+$ T cells *Proc. Natl. Acad. Sci. USA* **110**, E2046–E2053. doi:10.1073/pnas.1305227110
16. de Alwis, R. *et al*. (2016) Immunodominant dengue virus-specific $CD8^+$ T cell responses are associated with a memory PD-1^+phenotype. *J. Virol.* **90**, 4771–4779. doi:10.1128/JVI.02892-15
17. Zheng, H.Y. *et al*. (2020) Elevated exhaustion levels and reduced functional diversity of T cells in peripheral blood may predict severe progression in COVID-19 patients. *Cell. Mol. Immunol.* **17**, 541–543. doi:10.1038/s41423-020-0401-3
18. Grifoni, A. *et al*. (2020) Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. *Cell* **181**, 1489–1501. e15. doi:10.1016/j.cell.2020.05.015

Biographies

Dr Allison Imrie is an Associate Professor in the School of Biomedical Sciences, UWA. Her early work with human immunodeficiency virus (HIV) focussed on virus transmission between transmitter and recipient pairs, including transmission of antiviral drug resistant HIV species. She also characterised immune responses in early HIV infection. She was invited to work with the Global Program on AIDS in the World Health Organization Western Pacific Regional Office as a short-term consultant to advise member states to develop short-and medium-term plans for AIDS prevention and control and to assist with establishing HIV testing and surveillance programs. She then worked on mosquito-borne viral diseases of public health importance including dengue, and investigations of viral molecular epidemiology and immunopathogenesis. She has worked with colleagues in the Asia Pacific region to investigate neglected tropical diseases including dengue, Zika, Chikungunya and leptospirosis, and in Australia with her colleagues and students on endemic viruses including Ross River virus. She collaborates with her colleagues and students in Australia, China and the US to identify novel mosquito-borne viruses. Most recently she has been funded to investigate immune responses in people diagnosed with coronavirus infection.

Suzi McCarthy is Acting Medical Scientist in Charge of Microbiology at PathWest Laboratory Medicine in Perth, Western Australia where she oversees serological testing for Arboviruses and respiratory viruses (including COVID-19). Her research interests include developing and validating new diagnostic assays, and cell mediated immune responses to viral infections. In collaboration with the University of Western Australia, she has been investigating long term persistence of dengue virus-specific T-cell memory in Western Australian returning travellers with well defined monotypic dengue virus infection.

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Lipids, statins and susceptibility to SARS-CoV-2 and influenza A viruses

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Abstract. The extensive and on-going epidemiology studies of the SARS-CoV-2 pandemic have raised interesting observations on statins reducing COVID-19 severity. In this review, literature is analysed to examine how statins affect COVID-19 and influenza A, another pandemic respiratory virus. This information could be useful to prevent or reduce disease severity caused by respiratory viruses.

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The respiratory viruses, influenza A virus (IAV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have demonstrated their action to cause significant morbidity, mortality and socio-economic disruption. The 1918 influenza pandemic caused 20–100 million deaths, with one-third of the world's population being infected[1], while the current COVID-19 pandemic has resulted in 146 million confirmed cases and over 3 million deaths to date[2]. Our focus here is to review the potential for statins to affect patient outcomes for these viral infections.

Statins and cholesterol

Statins are among the most highly prescribed drugs used in the treatment of hypercholesterolemia, a major cause of cardiovascular disease. Diet has an effect on cholesterol levels, but our endogenous synthesis of cholesterol accounts for age-associated increases. To reduce plasma cholesterol to medically recommended levels of less than 4 mM, doctors prescribe statins to inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (Figure 1). Cholesterol is essential so it is important that statins do not block cholesterol synthesis completely. As shown in Figure 1, inhibition of HMGCR also affects other L-mevalonate pathways including protein prenylation[3]. Interestingly, statins can target any HMGCR, including HMGCRs of pathogenic *Candida* species and *Aspergillus fumigatus*[4].

Statins were discovered in the soil fungus *Aspergillus terreus*, which is currently used to produce lovastatin, a precursor of simvastatin. Simvastatin and atorvastatin were the first blockbuster drugs, but many additional statins have since been produced. Statins are used to inhibit HMGCR in the liver, reducing plasma cholesterol levels. Cholesterol is also an essential component of cell membranes, which become integrated into viral envelopes, leading us to review what is known about the effect of statins on SARS-CoV-2 and the other respiratory virus associated with pandemics, influenza A virus (IAV). Our findings are summarised in Table 1.

Cholesterol levels

Cholesterol is a vital part of IAV and SARS-CoV-2. During viral budding, lipids and cholesterol from infected host cells become part of the viral envelope[19]. Dietary cholesterol levels were shown to affect influenza infection in a mouse study[7]. Compared to a controlled diet group, mice with a 2% cholesterol diet experienced increased morbidity over a 5-week period.

The underlying low-grade chronic inflammation due to the release of the pro-inflammatory mediators from adipocytes of obese individuals exacerbates the cytokine storm observed in COVID-19 disease[20]. Obesity is also associated with the upregulation of ACE2 expression. ACE2 is a receptor for SARS-CoV-2 spike proteins, so its upregulation could further enhance viral attachment and entry to the host tissue and increase severity[21]. The higher content of lipid rafts with high cholesterol levels in obese patients may also support SARS-CoV-2 attachment to host cells and its subsequent replication. Importantly, cholesterol-rich lipid rafts in the host cell membrane are favourable for enveloped viruses making cholesterol reduction a general strategy to thwart enveloped virus infection[22].

Effect of statins

Statins have been investigated to determine whether they affect outcomes of IAV and SARS-CoV-2 infections. While benefits of atorvastatin and rosuvastatin have been demonstrated in a model of IAV infection in cell culture[14,15], the benefits to statin users have varied. A study comparing 5181 statin users with 5181 non-users found small benefits that were not statistically significant[16]. On the

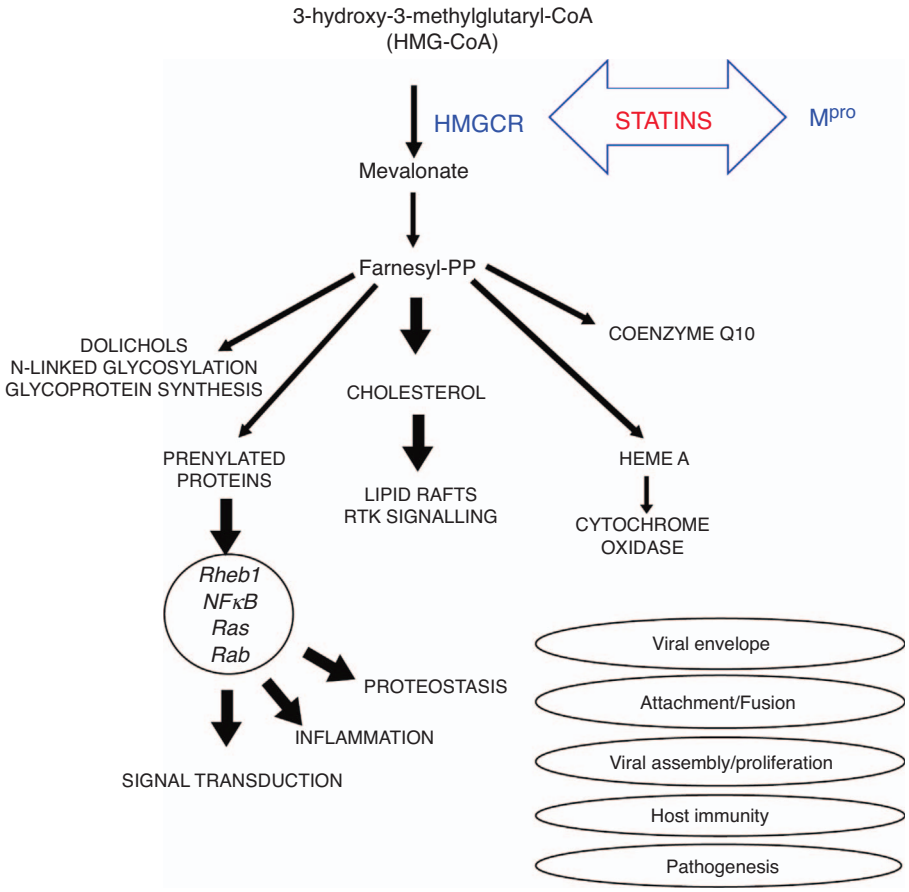

Figure 1. Molecular targets of statin treatment during SARS-CoV-2 infection showing inhibition of HMG-CoA reductase resulting in multiple effects.

Table 1. Effects of cholesterol and statins on SARS-CoV-2 and IAV infections.

	SARS-CoV-2	Influenza A virus
Effect of cholesterol/lipids	Host membrane cholesterol interacts with SARS-CoV-2 spike protein and facilitates viral entry to host cell[5] Dyslipidaemia is a common presentation in COVID-19 disease[5]	Envelope cholesterol is crucial for IAV entry and fusion to host cell membrane[6] Dietary cholesterol increased IAV infection in mice[7] Treatment with cholesterol lowering drugs significantly decreased IAV propagation in human airway epithelial cells[8]
Effect of statins	Statins reduced COVID-19 fatalities and severity by 30%[9] Use of statins reduced mortality due to COVID-19 in a retrospective observational study[10] Statin treatment in hospitalised COVID-19 patients reduced death rates and complications including acute kidney infection, sepsis and acute respiratory distress syndrome[11] Statin treatment reduced deaths due to COVID-19 in hospitalized patients[12] Potential binding with main protease (M^{pro}/NSP5), which is unique to and highly conserved in all coronaviruses[13]	Rosuvastatin and atorvastatin reduced IAV proliferation in kidney cells[14] Atorvastatin reduced IAV infection of MDK cells by >95%[15] A UK study showed a slight but not significant protection against hospitalisation and death in statin users[16] Statin usage in hospitalised patients with influenza was associated with reduced death rates[17] A moderate dose statin administration reduced the risk of death due to influenza and chronic obstructive pulmonary disease (COPD)[18]

other hand, in a large-scale matched cohort study (n = 76 232), moderate dose usage of statin was found beneficial by significantly reducing the risk of death due to COPD and influenza[18]. Similarly, in another multistate surveillance study, statin usage in patients hospitalised due to influenza was found associated with reduced mortality[17]. As influenza induces pro-inflammatory pathways by triggering the innate immune system, the anti-inflammatory pleotropic properties of statins have been studied to counteract it. Through *in vitro* tests, statins were able to inhibit IAV proliferation and possibly reduce inflammation by targeting Rho/Rho kinase pathways[14]. Several studies of patients with SARS-CoV-2 infection demonstrated the beneficial effects of statins, significantly reducing mortality rates and disease severity[9–12].

Mechanisms and thoughts on future therapeutics

It is now clear that statins have several additional effects apart from cholesterol synthesis inhibition which deserves further investigation.

SARS-CoV-2 main protease (M^{pro})

An *in silico* docking study demonstrated the potential of M^{pro}, the main protease of SARS-CoV-2, to bind a range of statins, possibly

explaining how statins can impede viral proliferation[13]. M^{pro} is essential for processing of the SARS-CoV-2 polyproteins[23]. Our BLASTP analyses show that sequences highly similar to SARS-CoV-2 M^{pro} are found in all other coronaviruses; however, they are absent in IAV (data available on request). The M^{pro} protein acts as dimer and its active site is composed of Cys-His dyad with the

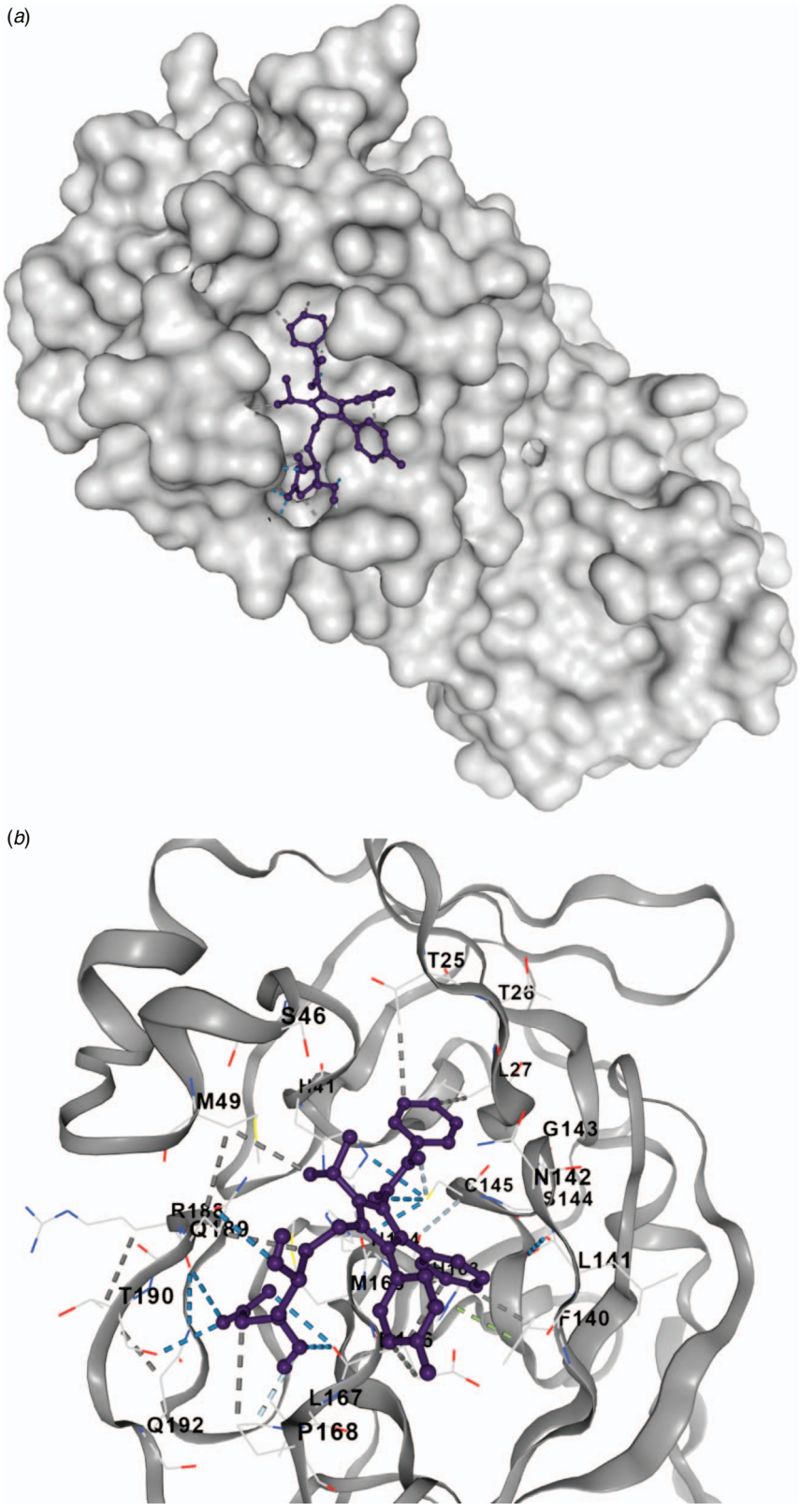

Figure 2. *In silico* docking analysis of SARS-CoV-2 M^{pro} structure 7JP1 (wild type structure retrieved from PDB database) with atorvastatin, performed using the CB-Dock online tool[30]. The binding of atorvastatin is shown at low (*a*) and high resolution (*b*). [Each of the statins, atorvastatin, fluvastatin, lovastatin, pitavastatin, rosuvastatin and simvastatin, bound M^{pro} at the catalytic Cys145 and His41 site with binding energies of –7.3, –7.1, –6.6, –6.9, –7.1 and –7.5 kcal/mol, respectively.]

Cys145 and His41 catalytic residues[24]. Our own *in silico* docking analysis of SARS-CoV-2 M^{pro} with statins demonstrates possible binding at the active site (including binding with Cys145 and His41) of the protease (Figure 2). This important knowledge may guide the design of better drugs to inhibit M^{pro} activity. The bodily distribution of statins is also important for drug targeting. To be effective, statins would need to reach the site of viral infection at levels sufficient for inhibition.

Proteostasis

Statins, like the lipophilic simvastatin, distribute widely in the body, and have additional effects like targeting protein turnover as well as providing an explanation of how simvastatin lowers the incidence of Alzheimer's disease[25,26]. One of the major effects of statin treatment is inhibition of protein prenylation by depleting farnesyl pyrophosphate and geranylgeranyl pyrophosphate. This reduction in protein prenylation also inhibits activation of proteins including Rheb1p, which in turn diminishes mammalian target of rapamycin (mTOR) mediated autophagy inhibition[27,28]. Such an effect of statin administration could enhance autophagy and associated lipolysis, which could further deplete intracellular lipids restricting the viral proliferation.

Inflammation

An additional effect of statin use is its ability to inhibit protein farnesylation, which causes adipogenesis arrest by lowering expression and activity of peroxisome proliferator activator gamma (PPARγ)[29]. Such interruption of adipocyte formation in statin users may lead to reduced release of pro-inflammatory markers, which has the potential to inhibit inflammation during COVID-19 infection[20]. Reduced protein prenylation due to statin treatment also produces an anti-inflammatory effect by inhibiting the activation of nuclear factor kappa B (NFκB)[27]. Another action of statins could include the effects on inflammation via the renin angiotensin system. The liver produces angiotensin that is converted to angiotensin I (inactive) by renin. The inactive angiotensin I is then converted to active angiotensin II, which plays a vital role in regulating inflammation, with the help of angiotensin converting enzyme 2 (ACE2). Angiotensin II, if acted on by ACE2, results in an anti-inflammatory effect. In contrast, angiotensin II interaction with the angiotensin II type 1 receptor (AT1R) proceeds towards release of pro-inflammatory mediators. However, an unhelpful effect of statins is the upregulation of ACE2 expression and the reduction of the pro-inflammatory pathway. On the contrary, overexpression of ACE2 due to statins may also potentially help SARS-CoV-2 viral entry to host[27].

Conclusion

Statins show promise in reducing severity of IAV and SARS-CoV-2, which could be attributed to inhibition HMGCR and a number of other targets. Specifically, the inhibition of protein prenylation has multiple effects including enhancing cytokine-induced inflammation, regulating proteostasis, and post-translational modifications of the intracellular proteins. These events are most likely to be involved in SARS-CoV-2 pathogenesis and viral proliferation as the virus utilises host machinery for survival and proliferation. Knowledge of the targeting of statins may improve the development of therapies for COVID-19 and IAV.

Conflicts of interest

Ian Macreadie is the Editor-in-Chief of *Microbiology Australia*, but was blinded from the peer-review process for this paper.

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Reference

1. Selleck, P. and Bernard, R. (2020) The 1918 Spanish influenza pandemic: *plus ça change, plus c'est la même chose*. *Microbiol. Aust.* **41**, 177–182. doi:10.1071/MA20049
2. WHO (2021) WHO Coronavirus (COVID-19) Dashboard. https://covid19.who.int/ (accessed 26 April 2021).
3. Zeiser, R. (2018) Immune modulatory effects of statins. *Immunology* **154**, 69–75. doi:10.1111/imm.12902
4. Westermeyer, C. and Macreadie, I.G. (2007) Simvastatin reduces ergosterol levels, inhibits growth and causes loss of mtDNA in *Candida glabrata*. *FEMS Yeast Res.* **7**, 436–441. doi:10.1111/j.1567-1364.2006.00194.x
5. Kočar, E. *et al*. (2021) Cholesterol, lipoproteins, and COVID-19: basic concepts and clinical applications. *Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids* **1866**, 158849. doi:10.1016/j.bbalip.2020.158849
6. Sun, X. and Whittaker, G.R. (2003) Role for influenza virus envelope cholesterol in virus entry and infection. *J. Virol.* **77**, 12543. doi:10.1128/JVI.77.23.12543-12551.2003
7. Louie, A.Y. *et al*. (2020) Dietary cholesterol affects the pathogenesis of influenza A virus infection in mice. *J. Immunol.* **204**, 93.19.
8. Bajimaya, S. *et al*. (2017) Cholesterol is required for stability and infectivity of influenza A and respiratory syncytial viruses. *Virology* **510**, 234–241. doi:10.1016/j.virol.2017.07.024
9. Kow, C.S. and Hasan, S.S. (2020) Meta-analysis of effect of statins in patients with COVID-19. *Am. J. Cardiol.* **134**, 153–155. doi:10.1016/j.amjcard.2020.08.004
10. Zhang, X.-J. *et al*. (2020) In-hospital use of statins is associated with a reduced risk of mortality among individuals with COVID-19. *Cell Metab.* **32**, 176–187.e4. doi:10.1016/j.cmet.2020.06.015
11. Torres-Peña, J.D. *et al*. (2021) Prior treatment with statins is associated with improved outcomes of patients with COVID-19: data from the SEMI-COVID-19 registry. *Drugs* **81**, 685–95.
12. Permana, H. *et al*. (2021) In-hospital use of statins is associated with a reduced risk of mortality in coronavirus-2019 (COVID-19): systematic review and meta-analysis. *Pharmacol. Rep.* 1–12.

13. Reiner, Ž. *et al*. (2020) Statins and the COVID-19 main protease: *in silico* evidence on direct interaction. *AMS* **16**, 490–496. doi:10.5114/aoms.2020.94655
14. Mehrbod, P. *et al*. (2014) Mechanisms of action and efficacy of statins against influenza. *BioMed Res. Int.* **2014**, 872370. doi:10.1155/2014/872370
15. Episcopio, D. *et al*. (2019) Atorvastatin restricts the ability of influenza virus to generate lipid droplets and severely suppresses the replication of the virus. *FASEB J.* **33**, 9516–9525. doi:10.1096/fj.201900428RR
16. Brassard, P. *et al*. (2017) The effect of statins on influenza-like illness morbidity and mortality. *Pharmacoepidemiol. Drug Saf.* **26**, 63–70. doi:10.1002/pds.4112
17. Vandermeer, M.L. *et al*. (2012) Association between use of statins and mortality among patients hospitalized with laboratory-confirmed influenza virus infections: a multistate study. *J. Infect. Dis.* **205**, 13–19. doi:10.1093/infdis/jir695
18. Frost, F.J. *et al*. (2007) Influenza and COPD mortality protection as pleiotropic, dose-dependent effects of statins. *Chest* **131**, 1006–1012. doi:10.1378/chest.06-1997
19. Musiol, A. *et al*. (2013) Annexin A6-balanced late endosomal cholesterol controls influenza A replication and propagation. *MBio* **4**, e00608-13. doi:10.1128/mBio.00608-13
20. Kim, J. and Nam, J.-H. (2020) Insight into the relationship between obesity-induced low-level chronic inflammation and COVID-19 infection. *Int. J. Obes.* **44**, 1541–1542. doi:10.1038/s41366-020-0602-y
21. Banerjee, M. *et al*. (2020) Obesity and COVID-19: a fatal alliance. *Indian J. Clin. Biochem.* **35**, 410–417. doi:10.1007/s12291-020-00909-2
22. Rawat, S.S. *et al*. (2003) Modulation of entry of enveloped viruses by cholesterol and sphingolipids *Mol. Membr. Biol.* **20**, 243–254. doi:10.1080/0968768031000104944
23. Dhakal, S. and Macreadie, I. (2021) Genes of SARS-CoV-2 and emerging variants. *Microbiol. Aust.* **42**, 10–12. doi:10.1071/MA21004
24. Kneller, D.W. *et al*. (2020) Structural plasticity of SARS-CoV-2 3CL M^{pro} active site cavity revealed by room temperature X-ray crystallography. *Nat. Commun.* **11**, 3202. doi:10.1038/s41467-020-16954-7
25. Dhakal, S. *et al*. (2019) Simvastatin efficiently reduces levels of Alzheimer's amyloid beta in yeast. *Int. J. Mol. Sci.* **20**, 3531. doi:10.3390/ijms20143531
26. Wolozin, B. *et al*. (2007) Simvastatin is associated with a reduced incidence of dementia and Parkinson's disease. *BMC Med.* **5**, 20. doi:10.1186/1741-7015-5-20
27. Subir, R. *et al*. (2020) Pros and cons for use of statins in people with coronavirus disease-19 (COVID-19). *Diabetes Metab. Syndr.* **14**, 1225–1229. doi:10.1016/j.dsx.2020.07.011
28. Basso, A.D. *et al*. (2005) The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits Rheb farnesylation and mTOR signaling: role in FTI enhancement of taxane and tamoxifen anti-tumour activity. *J. Biol. Chem.* **280**, 31101–31108. doi:10.1074/jbc.M503763200
29. Rivas, D. *et al*. (2007) Inhibition of protein farnesylation arrests adipogenesis and affects PPAR gamma expression and activation in differentiating mesenchymal stem cells. *PPAR Res.* **2007**, 81654. doi:10.1155/2007/81654
30. Liu, Y. *et al*. (2020) CB-Dock: a web server for cavity detection-guided protein–ligand blind docking. *Acta Pharmacol. Sin.* **41**, 138–144. doi:10.1038/s41401-019-0228-6

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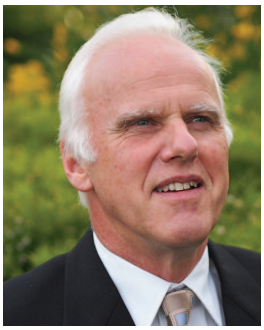

Ian Macreadie is an Honorary Professor at RMIT University and Editor-in-Chief of *Microbiology Australia*. His research and expertise are in diverse field of biosciences ranging from industrial microbiology to biomedical research.

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Helicobacteriology update

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Abstract. *Helicobacter pylori* colonises the gastric mucosa and is associated with various gastric diseases, including stomach cancer. At least 1 million new cases of stomach cancer cases are reported annually, and it is the fifth top cancer-killer in the world. Although *H. pylori* can be eradicated by a combination of antibiotics, the treatment success rate is declining due to the rise of antibiotic resistance. The same antibiotic combination must not be prescribed repeatedly. Susceptibility guided precision medicine is the most effective strategy to combat antibiotic resistant *H. pylori* cases. In addition, maintaining a stomach pH ≥6 during the antibiotic treatment is an important factor to increase cure rates. The new type of acid blocker, P-CABs, have shown promising results in *H. pylori* treatment. Natural products may suppress the *H. pylori* growth or relieve the symptoms but have not been successful in solving the root of the problem. New combination therapies show promise and the dream of 100% cure of the infection with minimal side effects from treatment seems achievable. The next decade will see combination therapies with newer acid blockers in widespread use at reasonable cost.

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Helicobacteriology update

Helicobacter pylori, or formally known as *Campylobacter pylori*, is a Gram-negative, micro-aerophilic, spiral microorganism that can colonise the healthy stomach lining. It is associated with gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer[1–3]. At least 50% of the world population is still infected with *H. pylori* and approximately 1 million new gastric cancer cases are reported annually[4]. In 1994 and 2017, the WHO classified *H. pylori* as a Class I carcinogen[5,6] and listed it as one of the most important (priority high) pathogens for emerging antibiotic resistance alarm[7,8], respectively.

It is interesting that 40 years after its discovery, with tens of thousands of research articles published, the route of transmission and the mechanism of how *H. pylori* causes cancer remains unclear. We now know that *H. pylori* survives poorly outside the human body. *In vitro*, *H. pylori* is known to be sensitive to heat, salt, chilli, honey, and many other common food ingredients[9]. This has made it difficult to transmit to other individuals via food sharing. However, people living under the same roof, with daily close contact, have been shown to infect each other[10]. On the flip side, we have also observed couples with good oral hygiene that have lived together for decades but have not infected each other. Perhaps a simple step in taking care of oral hygiene is sufficient in stopping *H. pylori* transmission.

The consensus is that we predominantly acquire *H. pylori* during childhood, perhaps via the oral-oral route, and traditionally from an infected mother to child. Whereas, in modern society, an infected father who shares the feeding duties could also be the source of infection. In situations where both parents must work, the caring duty may be given to either the grand-parents or nanny, who may be infected. Nevertheless, there is strong evidence to suggest that, as social economic status is improved, the prevalence of *H. pylori* declines.

Australia is one of the few countries that have a low prevalence of *H. pylori* (about 15%). However, the prevalence of *H. pylori* among the Aboriginal and the Asian communities can be as high as 50–80%[11,12]. We believe that the overall low prevalence of *H. pylori* is the major factor for the low gastric cancer incidence in Australia (7.3 cases per 100 000 persons; 10 for males and 4.5 for females)[13]. Gastric cancer may no longer be an Australian problem, but it is still the fifth top cancer-killer in the world[4,14]. Interestingly, about 50% of the newly reported gastric cancers are concentrated in the Eastern Asia countries, such as Japan, Korea, Mongolia and China[4]. Furthermore, all these Eastern Asia countries are dominated by the hpEAsia strain, and almost all of them harbour the more virulent EPIYA-D CagA toxin, noted oncoprotein[15,16]. CagA is arguably the most studied virulence factor of *H. pylori*. It is encoded on the 40 kb cag pathogenicity island and it is the only known effector protein to be injected into host cells[17]. CagA can lead to

inflammation[18], affecting the survival of B cells[19] and changes the histological characteristics of the stomach[20]. All these effects of CagA are thought to finally lead to the formation of gastric cancer.

From our past 20 years of clinical experience in culturing antibiotic resistant clinical *H. pylori* isolates, we have observed a growing number of multi-drug resistant *H. pylori* strains in the Western Australian population. For example, in our 2015 cohort, more than 20% of *H. pylori* isolates were triple drug resistant. It is believed that this is primarily due to a significant increase of migrants, especially from Asia. According to the 2011 census, 32.8% of Western Australia's population was born overseas (compared to the National average of 26.1%).

Presently, there is no standard treatment guideline for patients who carry antibiotic resistant *H. pylori* strains. Without the proper testing of antibiotic resistance in the laboratory, doctors are relying on experience and experimenting with different antibiotic combinations. This strategy may work for now, but we fear that it will only promote stronger antibiotic resistance in the future.

Thus, the best strategy for dealing with patients who failed multiple antibiotic treatment is to provide antibiotic susceptibility testing. Such personalised precision medicine has been proven to have high success rate. However, not every laboratory is capable of culturing such a fastidious microorganism. A robust and sensitive qPCR method to obtain quick antibiotic resistance diagnosis may be the alternative path overcoming the culturing hurdle. Besides, the only way of obtaining the *H. pylori* culture specimen is via endoscopy. Such a procedure can be difficult, costly, and is unavailable in the remote regions. Alternative technologies, such as the String Test that does not require medical specialists, should be investigated.

While the success rate of the standard *H. pylori* triple therapy (PPI + amoxicillin + metronidazole/clarithromycin) is declining globally, and is even abandoned in some countries, it remains effective in Australia[12]. Nevertheless, for those who failed the standard triple therapy, the alternative antibiotics used in rescue regimens include quinolones, rifampicin, tetracycline and furazolidone. While quinolones and rifampicin are effective antibiotics against *H. pylori*, the organism can be easily become resistant to these as well! Therefore, a better strategy in choosing antibiotic combination is required. To date, we still hear stories about patients who failed multiple times on the same treatment. It is important to remind doctors not to prescribe the same antibiotic combination to the patient who failed the *H. pylori* treatment, as the *H. pylori* must have already gained resistance to the treatment. Then again, amoxicillin resistance in *H. pylori* is rare. It is so rare that the mechanism of resistance is still unknown. As a result, should the patient not be allergic to penicillin, amoxicillin can be repeatedly used in subsequent *H. pylori* treatments. Luckily, resistance to tetracycline, furazolidone, and bismuth compound have not yet been reported. Bismuth compounds have been used in medicine for over three centuries and were first introduced to treat duodenal ulcer in 1987[21], but have gained more attention in recent years. Not only that, there are reports about overcoming metronidazole resistance by combination with tetracycline, but simply adding bismuth to triple therapy for 14 days has been reported to have an efficacy of more than 90%[22].

For many antibiotic treatments, the key factor to the success is the concurrent use of a high-dose proton-pump inhibitor (PPI). It is already known that the use of antibiotics alone is not enough to eradicate *H. pylori*. Acid reduction therefore plays a vital role in *H. pylori* treatment. To elaborate on this, the reader should note that most antibiotics were developed without the gastric mucosa in mind. Therefore, they might not act in the gastrointestinal lumen, and especially not in an acid environment. Interestingly, metronidazole and clarithromycin, which are secreted in saliva, are particularly effective against naïve *H. pylori* strains, perhaps for this reason. Bismuth compound acts topically on the gastric mucosa and is safe and effective (used for at least 200 years for gastrointestinal disorders). However, bismuth does not penetrate the mucus layer so always needs an extra antibiotic agent to provide a permanent cure.

Regarding acid-lowering agents, one aims to achieve around the clock pH $\geq$6 in the stomach. H_2 blockers (e.g. cimetidine 'Tagamet', ranitidine 'Zantac', famotidine 'Calmicidetc') are competitive inhibitors of acid secretion so cannot reliably do this. The PPI drugs were a breakthrough in this regard (e.g. omeprazole 'Losec', esomeprazole 'Nexium', rabeprazole 'Aciphex') almost completely blocking the proton pumps. However, some *H. pylori* could survive, perhaps reflecting an inadequate dose in some patients.

Recently, the potassium competitive acid blocker group (P-CABs) has been used (Vonoprazan), which might give a rapid and more complete acid blockade, with subsequent excellent cure rate for *H. pylori*. Perhaps even with just a single antibiotic such as amoxicillin. Time will tell!

But the controversy still rages, 'should we give treatment to asymptomatic *H. pylori* carriers?' Asymptomatic patients with a family history of gastric cancer, or with gastric intestinal metaplasia, or atrophic gastritis, are advised to get rid of their *H. pylori*. In regions with high prevalence of gastric cancer, such as Eastern Asia, where the 'cancer strain' of *H. pylori* predominates, should all be encouraged to eradicate the *H. pylori* infection? The risk of getting gastric cancer increases with age. Since most people acquire *H. pylori* during childhood, and assuming that the damage of the gastric mucosa accumulates through age, the chance of developing gastric cancer increases. Perhaps because the seeds of cancer have already been planted, getting rid of *H. pylori* in old age does not

always eliminate the gastric cancer risk. However, it has been reported that in all age groups, patients with a history of *H. pylori* infection have a higher risk of gastric cancer than those that have never been infected[23–25].

Certainly, *H. pylori* only colonises the internal gastric luminal surfaces, albeit under the mucus layer. Therefore, it is exposed to any food or medicine ingested by the host. So, this is a perceived vulnerability for *H. pylori*. The 'Holy Grail' of *H. pylori* treatment is hence the discovery of orally active natural or food products which might cure the infection, or perhaps suppress it enough to allow natural immune processes to finish the task. Alas, at the present time, most natural products show zero effect on *H. pylori*, or at best, a weak temporary effect. Often, *H. pylori* tenderfoots will be excited about *in vitro* killings of *H. pylori*, but this hardly ever translates into useful clinical activity.

In summary then, susceptibility guided precision medicine is the way forward for eradication of *H. pylori*. New combination therapies show promise and the dream of 100% cure of the infection with minimal side effects from treatment seems achievable. The next decade will see combination therapies with newer acid blockers in widespread use at reasonable cost. Investment in new antibiotics and strategies to combat the rise of antibiotic resistant microorganisms is vital. The famous quote by Dr David Graham 'The only good *Helicobacter pylori* is a dead *Helicobacter pylori*'[26] seems the way to go.

Conflicts of interest

Barry Marshall is medical director of Tri-Med (http://www.trimed.com.au), a Perth company that distributes diagnostic tests for *Helicobacter pylori* ('PYtest' urea breath tests and 'CLOtest' biopsy rapid urease test), and marketing orphan drugs (bismuth subcitrate, tetracycline, furazolidone and rifaximin). Alfred Tay and Michael Wise declare no conflicts of interest.

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References

1. Labigne, A. and de Reuse, H. (1996) Determinants of *Helicobacter pylori* pathogenicity. *Infect. Agents Dis.* **5**, 191–202.
2. McColl, K.E. (1997) *Helicobacter pylori*: clinical aspects. *J. Infect.* **34**, 7–13. doi:10.1016/S0163-4453(97)80003-5
3. Marshall, B.J. and Warren, J.R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **323**, 1311–1315. doi:10.1016/S0140-6736(84)91816-6
4. Bray, F. *et al*. (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **68**, 394–424. doi:10.3322/caac.21492
5. (1994) Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7–14 June 1994. *IARC Monogr. Eval. Carcinog. Risks Hum.* **61**, 1–241.
6. Moss, S.F. (2017) The clinical evidence linking *Helicobacter pylori* to gastric cancer. *Cell. Mol. Gastroenterol. Hepatol.* **3**, 183–191. doi:10.1016/j.jcmgh.2016.12.001
7. Tacconelli, E. *et al*. (2018) Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* **18**, 318–327. doi:10.1016/S1473-3099(17)30753-3
8. Dang, B.N. and Graham, D.Y. (2017) *Helicobacter pylori* infection and antibiotic resistance: a WHO high priority? *Nat. Rev. Gastroenterol. Hepatol.* **14**, 383–384. doi:10.1038/nrgastro.2017.57
9. O'Mahony, R. *et al*. (2005) Bactericidal and anti-adhesive properties of culinary and medicinal plants against *Helicobacter pylori*. *World J. Gastroenterol.* **11**, 7499–7507. doi:10.3748/wjg.v11.i47.7499
10. Sari, Y.S. *et al*. (2008) *H pylori*: treatment for the patient only or the whole family? *World J. Gastroenterol.* **14**, 1244–1247. doi:10.3748/wjg.14.1244
11. Windsor, H.M. *et al*. (2005) Prevalence of *Helicobacter pylori* in Indigenous Western Australians: comparison between urban and remote rural populations. *Med. J. Aust.* **182**, 210–213. doi:10.5694/j.1326-5377.2005.tb06668.x
12. Wise, M.J. *et al*. (2019) A longitudinal, population-level, big-data study of *Helicobacter pylori*-related disease across Western Australia. *J. Clin. Med.* **8**, 1821. doi:10.3390/jcm8111821
13. Australian Institute of Health and Welfare (2020) Stomach cancer statistics. https://www.canceraustralia.gov.au/affected-cancer/cancer-types/stomach-cancer/stomach-cancer-statistics (accessed March 2021).
14. Sung, H. *et al*. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* doi:10.3322/caac.21660
15. Higashi, H. *et al*. (2002) Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc. Natl. Acad. Sci. USA* **99**, 14428–14433. doi:10.1073/pnas.222375399
16. Olbermann, P. *et al*. (2010) A global overview of the genetic and functional diversity in the *Helicobacter pylori cag* pathogenicity island. *PLoS Genet.* **6**, e1001069. doi:10.1371/journal.pgen.1001069
17. Censini, S. *et al*. (1996) cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. USA* **93**, 14648–14653. doi:10.1073/pnas.93.25.14648
18. Posselt, G. *et al*. (2013) The functional interplay of *Helicobacter pylori* factors with gastric epithelial cells induces a multi-step process in pathogenesis. *Cell Commun. Signal.* **11**, 77. doi:10.1186/1478-811X-11-77
19. Umehara, S. *et al*. (2003) Effects of *Helicobacter pylori* CagA protein on the growth and survival of B lymphocytes, the origin of MALT lymphoma. *Oncogene* **22**, 8337–8342. doi:10.1038/sj.onc.1207028
20. Murata-Kamiya, N. *et al*. (2007) *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* **26**, 4617–4626. doi:10.1038/sj.onc.1210251
21. Marshall, B.J. *et al*. (1987) Antibacterial action of bismuth in relation to *Campylobacter pyloridis* colonization and gastritis. *Digestion* **37**, 16–30. doi:10.1159/000199555
22. McNicholl, A.G. *et al*. (2020) Combination of bismuth and standard triple therapy eradicates *Helicobacter pylori* infection in more than 90% of patients. *Clin. Gastroenterol. Hepatol.* **18**, 89–98. doi:10.1016/j.cgh.2019.03.048
23. Kumar, S. *et al*. (2020) Risk factors and incidence of gastric cancer after detection of *Helicobacter pylori* infection: a large cohort study. *Gastroenterology* **158**, 527–536 e7. doi:10.1053/j.gastro.2019.10.019
24. Leung, W.K. *et al*. (2018) Effects of *Helicobacter pylori* treatment on incidence of gastric cancer in older individuals. *Gastroenterology* **155**, 67–75. doi:10.1053/j.gastro.2018.03.028
25. Choi, I.J. *et al*. (2020) Family history of gastric cancer and *Helicobacter pylori* treatment. *N. Engl. J. Med.* **382**, 427–436. doi:10.1056/NEJMoa1909666
26. Graham, D.Y. (1997) The only good *Helicobacter pylori* is a dead *Helicobacter pylori*. *Lancet* **350**, 70–71. doi:10.1016/S0140-6736(05)66278-2

Biographies

Dr Chin Yen Tay has great interest in clinical *Helicobacter pylori* research, its genomics and next generation sequencing technology. For the past 5 years, Dr Tay has been profiling and monitoring the antibiotic resistant pattern of *H. pylori* in Western Australia. Since *H. pylori* has strong association with gastric cancer, and China is a gastric cancer hotspot, many doctors in China are still under tremendous stress for not able to eradicate *H. pylori* infection due to antibiotic resistance. Moreover, only a handful of laboratories in China is capable of culturing *H. pylori*. Therefore, in 2017, via a successful grant application with Shenzhen Dapeng Hospital, Dr Tay was given a supportive grant from Shenzhen Dapeng Hospital to help develop capability in *H. pylori* research. Dr Tay has showed that the culturing technique is transferable and in 2018, funded by the Australian-China Councils Fellowship, has allowed him to provide a one-month training program in University of Western Australia, Helicobacter Research Laboratory. Dr Tay is hoping to use this opportunity to collaborate with more hospitals and research institutes in China to gain more understanding of the China *H. pylori* strain.

Professor Michael Wise completed a double degree in Engineering and Arts and a PhD in electrical engineering at the University of New South Wales. He then worked for the University Technology, Sydney for two years before lecturing in Computer Science at the University of Sydney. Here he created computer software for use in plagiarism detection until the discovery that his programs had a secondary use in gene sequence alignment prompted him to shift his research to bioinformatics. Prof Wise was subsequently employed a Senior Research Fellow at Pembroke College in Cambridge. In 2004 he moved to The University of Western Australia, where he had a joint appointment in the School of Biomedical, Biomolecular and Chemical Sciences and in Computer Science and Software Engineering. Since July 2016, he is now solely in Computer Science and Software Engineering.

Professor Barry Marshall has managed a *Helicobacter pylori* research group almost since his discovery of *H. pylori* with Robin Warren in 1982. Professor Marshall has received many honours for his work on *Helicobacter pylori*. Most notably they include the Nobel Prize for Medicine or Physiology in 2005, The William Beaumont Prize in 2006 (American Gastroenterology Association), in 1995 the Albert Lasker Award (Albert & Mary Lasker Foundation), and in 1998 the Dr A. H. Heineken Prize for Medicine (The Alfred Heineken Foundation, Amsterdam). In 2007 Professor Marshall was awarded the honour of Western Australian of the year and The Companion in the General Division of the Order of Australia (AC). In the past few years Professor Marshall's laboratory has developed enhanced methods for non-invasive studies on the molecular epidemiology of *H. pylori*, notably rapid breath test methods and retrieval of the organism (and culture) from a swallowed string. Professor Marshall has also recently succeeded in drawing investor funding from international and national sources for the creation of Ondek; a small biotech company focused on developing new biologic delivery systems for vaccines and therapeutics, utilising the unique characteristics of genetically modified *H. pylori*.

Breaking Research

Call for Expressions of Interest

Early career (less than 5 year's post-graduation) and student researchers who would like their area of research to be featured in *Microbiology Australia* are invited to contribute a proposal of their articles and its impact.

The Editorial Board will select up to 10 articles for invited submissions. Articles will be peer reviewed and feature in the third issue of 2021.

As a guide, the article should be up to 1500 words, be targeted to the wider community of Australian microbiologists and should describe the author's original research. Articles will go through the normal process of peer review and editing.

Please send Expressions of Interest (EOI) to editorasm@gmail.com before July 2021 and include suggested title, name of contributor and contact details (name of supervisor for students and a brief CV listing graduation year for early career researchers), and a brief abstract of less than 200 words.

All contributors must be members of the *Australian Society for Microbiology*.

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Melioidosis in Australia

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Abstract. Melioidosis is a potentially fatal bacterial infection caused by the Gram-negative bacillus, *Burkholderia pseudomallei* following contact with a contaminated environmental source, normally soil or water in tropical and subtropical locations. The disease spectrum varies from rapidly progressive bacteraemic infection with or without pneumonia, to focal lesions in deep soft tissues and internal organs to superficial soft tissue infection and asymptomatic seroconversion with possible long-term dormancy. Most infections occur with a background of chronic illness such as diabetes, chronic kidney disease and alcoholic liver disease. Improvements in diagnosis, targeted antimicrobial treatment and long term follow up have improved clinical outcomes. Environmental controls following rare point source case clusters and heightened awareness of melioidosis appear to have reduced the disease burden in some parts of northern Australia. However, the impact of climate change on dispersal of environmental *B. pseudomallei,* and changing land use in tropical Australia is expected to change the epidemiology of melioidosis in future.

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Introduction

Melioidosis has fascinated Australian microbiologists since it was first encountered as a human infection in a 32-year-old Townsville man with diabetes in 1950[1]. Human melioidosis has a remarkable ability to cause a broad spectrum of human disease from rapidly fatal bacteraemic infection and necrotising pneumonia, through persistent localised chronic lesions to asymptomatic dormant infections that convert to more serious infection after intervals of months to years[2]. As a consequence, melioidosis challenges the logic of conventional clinico-pathological disease classifications and is best considered as a cluster of syndromes linked by a single bacterial aetiology, the Gram-negative, oxidase positive bacillus, *Burkholderia pseudomallei* and a history of environmental exposure.

Epidemiology

Melioidosis in Australia is endemic across the tropical north of Australia (Figure 1) where it occurs as a sporadic infection of people who have had exposure to contaminated soil or surface water through direct transdermal inoculation, inhalation and possibly ingestion[3]. Occasional point source case clusters have occurred related to contaminated water, medical solutions or hand wash products[4–7], and animal case clusters have been associated with flooding of pasture land[8]. The majority of acute febrile melioidosis occurs as a septicaemia with or without pneumonia, peaking during the tropical wet season, and sometimes follows in the wake of tropical cyclones[9], though not necessarily in all of northern Australia[10]. However, the potentially long symptom-free period leads to some subacute infections presenting during the dry season, or in residents of temperate Australia who previously travelled to the tropics[11], including endemic locations overseas. There is a higher risk of bacteraemic infections in people with one or more of a group of co-morbidities, most notably diabetes, chronic lung disease, chronic kidney disease and alcoholic liver disease[12].

Diagnosis

The lack of pathognomonic clinical features, wide range of clinical presentations and potentially dormant deep soft tissue infections create difficulties for the diagnosing physician. In patients from tuberculosis-endemic settings, there is a risk of misdiagnosis of melioidosis as tuberculosis and subsequent inappropriate treatment[13]. Attempts have been made to standardise clinical definitions of melioidosis[14]. Other than in the few centres in tropical Australia that encounter enough cases to gain experience applying such classification, maintaining awareness of melioidosis is more easily said than done. The most reliable laboratory confirmation comes from isolating *B. pseudomallei* in blood, sputum, abscess fluid or other culture[15] (Figure 2). However, confirmation of the identity of *B. pseudomallei* can be challenging in clinical laboratories that have not previously handled the species. Referral of a suspect isolate

(Gram-negative bacillus, oxidase positive, Gentamicin and Colistin resistant) to a reference laboratory may be needed, although this will add further delays to reporting results. Laboratories in the melioidosis endemic zone will often use advanced bacterial identification methods such as specific PCR assays, MALDI-TOF mass spectrophotometry or gene sequencing to produce a definitive identification[17,18]. Serological assays are used as a complementary diagnostic method, particularly in the absence of a positive culture, but background antibody levels in endemic regions may confound result interpretation[19]. Moreover, high risk exposure activities that result in confirmed infection do not necessarily cause seroconversion[20].

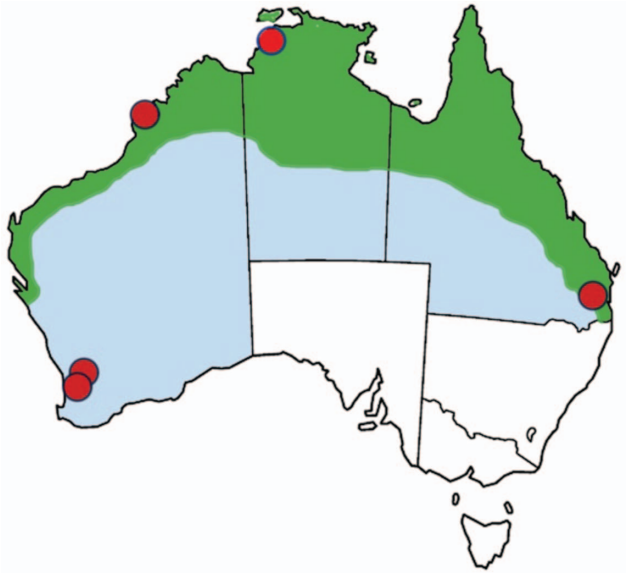

Figure 1. Melioidosis distribution in Australia (blue), and the main endemic area (green), showing the location of case clusters (red spots).

Treatment

Detailed treatment regimens can be found in the Therapeutic Guidelines and are updated periodically by specialists with current clinical experience[21]. In summary, acute bacteraemic and other severe infections are treated with an intravenous beta-lactam agent such as Ceftazidime or Meropenem in an intensive phase for between 2 and 8 weeks, then followed by an extended period of eradication with one or more oral antimicrobial agents to counter the risk of relapse in an eradication phase lasting 3–6 months. The revised guidelines vary with presence and location of focal disease. Control of co-morbid conditions such as diabetes and chronic kidney disease during this eradication phase is likely to be an important contributor to eventual success of eradication therapy, but can be confounded by poor compliance with oral treatment regimens[22]. The restricted range of antimicrobial agents effective against *B. pseudomallei* reflects its natural habitat as a soil-dwelling bacterium, where it has evolved a range of mechanisms for antibiotic inactivation, notable among these being a collection of efficient efflux pumps[23]. Though the success of the Darwin treatment protocol is clear from improved treatment outcomes, high levels of intrinsic antimicrobial resistance and concerns about emerging acquired resistant have prompted the application of genomics to predict antimicrobial resistance in *B. pseudomallei*[24]. Moreover, the ability of *B. pseudomallei* to sequestrate in cells and tissues where

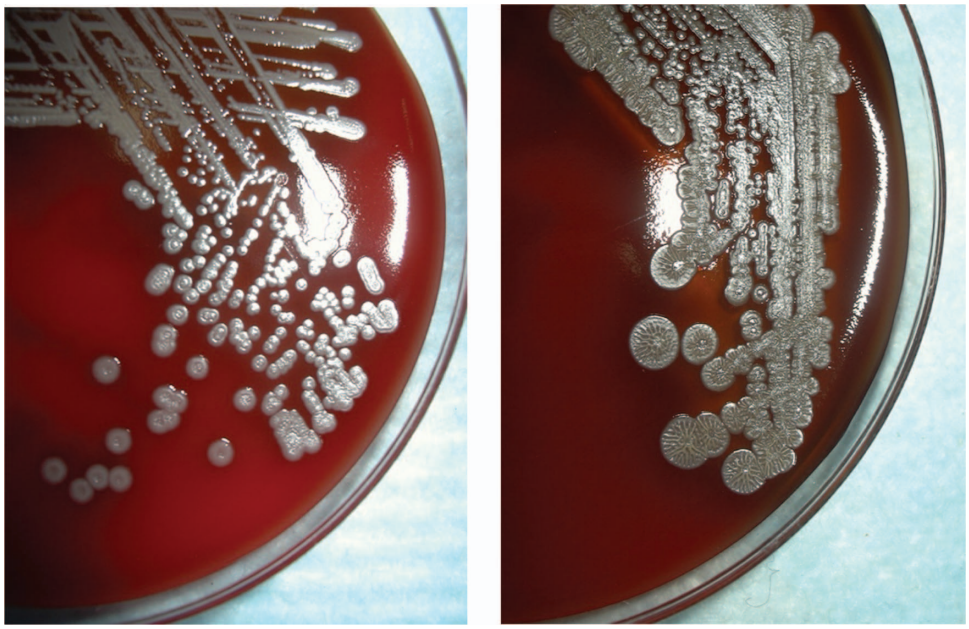

Figure 2. *B. pseudomallei* growth on horse blood agar after 24 (left) and 48 h (right), demonstrating the development of wrinkled colony appearance. Some strains do not wrinkle at all. An earthy odour is often noticed when agar plates are opened, due to the production of volatile organic compounds[16].

antimicrobial bioavailability is poor presents a challenge to guaranteeing effective intracellular antimicrobial activity.

Pathogenesis

The unusually broad range of clinical presentations of melioidosis have yet to be fully explained at a mechanistic level, but it is becoming clear that virulence of infection is predominantly a function of host risk factors[25]. *B. pseudomallei* is a facultative intracellular bacterial pathogen capable of entry into and prolonged survival within professional phagocytic cells[26]. Like other facultative intracellular bacteria associated with infections of public health interest, *B. pseudomallei* deploys a range of molecular mechanisms that likely reflect its evolutionary history as a soil-dwelling species. Indeed, its ability to invade and persist in naturally occurring soil microbiota such as free-living amoebae suggest a possible environmental origin for its cellular virulence[27]. However, a subset of *B.pseudomallei* possess a *Burkholderia mallei*-like sequence variation in the actin-based motility gene whose presence correlates with rapid dissemination and replication at a range of locations including the nervous system and thus have a molecular basis for neurotropism[28].

Genomics

The explosion of microbial genomics has led to important insights into the molecular biology and immunology of melioidosis. Whole genome sequencing indicates that *B. pseudomallei* has one of the largest known bacterial genomes at around 6.5 Mb, arranged in two chromosomes of unequal size[29]. The operons associated with virulence are mainly located on the smaller of the two. Recent phylogeographic analysis indicates that the Southeast Asian clade arose from an ancient Australian clade, which may have early remnants in Papua New Guinea and the Torres Strait islands[30]. Non-pathogenic near neighbour species such as *Burkholderia ubonensis* and *Burkholderia thailandensis* have also been found in pristine wilderness locations during *B. pseudomallei* environmental survey work, raising questions about the phylogeographic origins of the wider *B. pseudomallei* group[31]. At a more pragmatic level, genotyping studies have been instrumental in confirming single points of origin for melioidosis case clusters[4–7] and have shown the plausibility of occasional long-distance translocation of *B. pseudomallei* strains associated with human infection[9].

A changing public health threat

All Australian jurisdictions in the tropics have made melioidosis a notifiable infection. Following controls applied in the aftermath of the Western Australian case cluster in 1997[32], bacteraemic melioidosis is now rare in WA and almost eliminated in our indigenous population. The majority of cases are in long distance travellers[11] and even these have fallen recently due to pandemic travel restrictions. However, the recent Southwestern WA case cluster was a stark reminder of the greater difficulty detecting *B. pseudomallei* soft tissue infections[6], particularly when geographic location, clinical presentation and exposure history are unexpected. We have to ask how many subacute and initially asymptomatic infections are missed. Noting the association with cyclone tracks, and the changing patterns of cyclone behaviour as a consequence of climate change[9], we should be alert to the possibility of an extension of the Australian melioidosis endemic zone. The increased political instability of our region due to the effects of the COVID pandemic should also alert us to the deliberate dissemination of *B. pseudomallei*. This may seem far-fetched, but was under active consideration in the wake of anthrax spore/white powder events in 2001.

Conclusion

Melioidosis is a disease complex attributed to a multi-competent Gram-negative bacillus, *B. pseudomallei.* High rates of mortality in acute melioidosis survivors remain an unresolved problem[33]. The clinical and scientific experience built up in Australian centres of excellence, particularly in our tropics, has advanced diagnosis, treatment and prevention of severe and subacute disease variants. However, the natural environmental habitat of *B. pseudomallei* ensures that the principal reservoir of human infection cannot be eliminated. Changing patterns of land use, human encounter with environmental *B. pseudomallei*, and environmental influences like climate change guarantee further challenges for Australian microbiologists in years to come.

Conflicts of interest

The author declares no conflicts of interest.

Declaration of funding

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References

1. Rimington, R.A. (1962) Melioidosis in north Queensland. *Med. J. Aust.* **1**, 50–53. doi:10.5694/j.1326-5377.1962.tb76106.x
2. Gassiep, I. *et al*. (2020) Human melioidosis. *Clin. Microbiol. Rev.* **33**, e00006-19. doi:10.1128/CMR.00006-19
3. Currie, B.J. and Kaestli, M. (2016) Epidemiology: a global picture of melioidosis. *Nature* **529**, 290–291. doi:10.1038/529290a
4. Inglis, T.J. *et al*. (2000) *Burkholderia pseudomallei* traced to water treatment plant in Australia. *Emerg. Infect. Dis.* **6**, 56–59. doi:10.3201/eid0601.000110

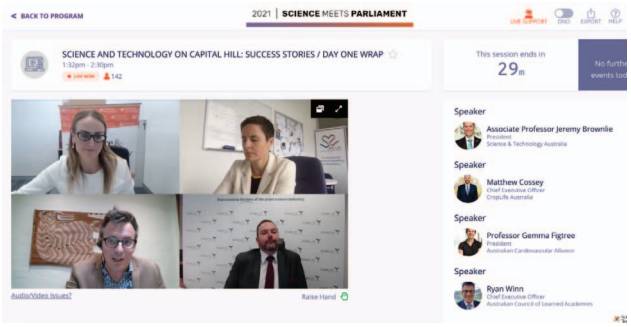
5. Currie, B.J. *et al*. (2001) A cluster of melioidosis cases from an endemic region is clonal and is linked to the water supply using molecular typing of *Burkholderia pseudomallei* isolates. *Am. J. Trop. Med. Hyg.* **65**, 177–179. doi:10.4269/ajtmh.2001.65.177
6. Merritt, A.J. *et al*. (2016) Cutaneous melioidosis cluster caused by contaminated wound irrigation fluid. *Emerg. Infect. Dis.* **22**, 1420–1427. doi:10.3201/eid2208.151149
7. Gal, D. *et al*. (2004) Contamination of hand wash detergent linked to occupationally acquired melioidosis. *Am. J. Trop. Med. Hyg.* **71**, 360–362. doi:10.4269/ajtmh.2004.71.360
8. Webb, J.R. *et al*. (2020) A persisting nontropical focus of *Burkholderia pseudomallei* with limited genome evolution over five decades. *mSystems* **5**, e00726-20. doi:10.1128/mSystems.00726-20
9. Merritt, A.J. and Inglis, T.J.J. (2017) The role of climate in the epidemiology of melioidosis. *Curr. Trop. Med. Rep.* **4**, 185–191. doi:10.1007/s40475-017-0124-4
10. Stewart, J.D. *et al*. (2017) Melioidosis in Far North Queensland is not correlated with severe weather events. *Med. J. Aust.* **207**, 394. doi:10.5694/mja16.01332
11. Dan, M. (2015) Melioidosis in travelers: review of the literature. *J. Travel Med.* **22**, 410–414. doi:10.1111/jtm.12236
12. Currie, B.J. *et al*. (2004) Melioidosis epidemiology and risk factors from a prospective whole-population study in northern Australia. *Trop. Med. Int. Health* **9**, 1167–1174. doi:10.1111/j.1365-3156.2004.01328.x
13. Garg, R. *et al*. (2020) Melioidosis in suspected recurrent tuberculosis: a disease in disguise. *J. Infect. Dev. Ctries.* **14**, 312–316. doi:10.3855/jidc.12051
14. Cheng, A.C. (2010) Melioidosis: advances in diagnosis and treatment. *Curr. Opin. Infect. Dis.* **23**, 554–559. doi:10.1097/QCO.0b013e32833fb88c
15. Fairley, L. *et al*. (2021) Systematic review and meta-analysis of diagnostic tests for diagnosis of melioidosis. *Acta Trop.* **214**, 105784. doi:10.1016/j.actatropica.2020.105784
16. Inglis, T.J. *et al*. (2015) Volatile-sulfur-compound profile distinguishes *Burkholderia pseudomallei* from *Burkholderia thailandensis*. *J. Clin. Microbiol.* **53**, 1009–1011. doi:10.1128/JCM.03644-14
17. Gassiep, I. *et al*. (2021) Diagnosis of melioidosis: the role of molecular techniques. *Future Microbiol.* **16**, 271–288. doi:10.2217/fmb-2020-0202
18. Inglis, T.J. *et al*. (2012) Use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis for rapid confirmation of *Burkholderia pseudomallei* in septicemic melioidosis. *Am. J. Trop. Med. Hyg.* **86**, 1039–1042. doi:10.4269/ajtmh.2012.11-0454
19. Chaichana, P. *et al*. (2018) Antibodies in melioidosis: the role of the indirect hemagglutination assay in evaluating patients and exposed populations. *Am. J. Trop. Med. Hyg.* **99**, 1378–1385. doi:10.4269/ajtmh.17-0998
20. Grivas, R. *et al*. (2015) A prospective study of melioidosis after environmental exposure of healthy participants to *Burkholderia pseudomallei* during a muddy endurance challenge. *Am. J. Trop. Med. Hyg.* **92**, 773–775. doi:10.4269/ajtmh.14-0756
21. Sullivan, R.P. *et al*. (2020) 2020 Review and revision of the 2015 Darwin melioidosis treatment guideline; paradigm drift not shift. *PLoS Negl. Trop. Dis.* **14**, e0008659. doi:10.1371/journal.pntd.0008659
22. Stewart, J.D. *et al*. (2017) The epidemiology and clinical features of melioidosis in Far North Queensland: implications for patient management. *PLoS Negl. Trop. Dis.* **11**, e0005411. doi:10.1371/journal.pntd.0005411
23. Krishnamoorthy, G. *et al*. (2019) Efflux pumps of *Burkholderia thailandensis* control the permeability barrier of the outer membrane. *Antimicrob. Agents Chemother.* **63**, e00956-19. doi:10.1128/AAC.00956-19
24. Madden, D.E. *et al*. (2021) Taking the next-gen step: comprehensive antimicrobial resistance detection from B*urkholderia pseudomallei*. *EBioMedicine* **63**, 103152. doi:10.1016/j.ebiom.2020.103152
25. Webb, J.R. *et al*. (2019) *Burkholderia pseudomallei* lipopolysaccharide genotype does not correlate with severity or outcome in melioidosis: host risk factors remain the critical determinant. *Open Forum Infect. Dis.* **6**, ofz091. doi:10.1093/ofid/ofz091
26. Whiteley, L. *et al*. (2017) Entry, intracellular survival, and multinucleated-giant-cell-forming activity of *Burkholderia pseudomallei* in human primary phagocytic and nonphagocytic cells. *Infect. Immun.* **85**, e00468-17. doi:10.1128/IAI.00468-17
27. Inglis, T.J. *et al*. (2000) Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* species results in coiling phagocytosis, endamebic bacterial survival, and escape. *Infect. Immun.* **68**, 1681–1686. doi:10.1128/IAI.68.3.1681-1686.2000
28. Morris, J.L. *et al*. (2017) Increased neurotropic threat from *Burkholderia pseudomallei* strains with a *B. mallei*-like variation in the *bimA* motility gene, Australia. *Emerg. Infect. Dis.* **23**, 740–749. doi:10.3201/eid2305.151417
29. Songsivilai, S. and Dharakul, T. (2000) Multiple replicons constitute the 6.5-megabase genome of *Burkholderia pseudomallei*. *Acta Trop.* **74**, 169–179. doi:10.1016/S0001-706X(99)00067-4
30. Baker, A.L. *et al*. (2018) *Burkholderia pseudomallei* distribution in Australasia is linked to paleogeographic and anthropogenic history. *PLoS One* **13**, e0206845. doi:10.1371/journal.pone.0206845
31. Levy, A. *et al*. (2008) Expanded range of *Burkholderia* species in Australia. *Am. J. Trop. Med. Hyg.* **78**(4), 599–604. doi:10.4269/ajtmh.2008.78.599
32. Inglis, T.J. *et al*. (2011) The aftermath of the Western Australian melioidosis outbreak. *Am. J. Trop. Med. Hyg.* **84**, 851–857. doi:10.4269/ajtmh.2011.10-0480
33. Hanson, J. and Smith, S. (2019) High rates of premature and potentially preventable death among patients surviving melioidosis in tropical Australia. *Am. J. Trop. Med. Hyg.* **101**, 328–331. doi:10.4269/ajtmh.19-0375

Biography

Dr Inglis is Head of Pathology and Laboratory Medicine at the University of Western Australia, and appointed as a Medical Microbiologist at PathWest Laboratory Medicine WA. He qualified in Medicine at the University of Southampton, and followed this with a Doctor of Medicine thesis on ventilator associated pneumonia. After three years in Singapore where he first encountered melioidosis, he moved to Western Australia to take up his position with the state pathology service. Shortly afterwards he investigated the Kimberley melioidosis outbreak and its aftermath, completing a PhD on the environmental biology of *Burkholderia pseudomallei*. Since then he has expanded on laboratory biopreparedness, regional pathology capability building and the laboratory response to emerging infectious diseases, including the current pandemic.

Science meets Parliament 2021

Ulrike Kappler
Chair of Queensland State Branch, ASM



News of changes to funding for higher education and the STEM sector, budget cuts and redistribution of government funding for particular university subjects are common, and anyone who has applied for major grant funding is familiar with national priority areas for research. While these are vital decisions that impact anyone working in the sector, I only had a rough idea of what influences these decisions and how they are taken when I was offered the opportunity to attend Science meets Parliament 2021 as an ASM delegate.

Science meets Parliament (SMP) is the annual flagship event of Science & Technology Australia (STA), the Federation of Australian Scientific and Technological Societies, and brings together Australian scientists, technologists, parliamentarians, journalists and policy-makers, all with the aim of fostering dialogue between these parties and increasing the visibility of science in day-to-day political agendas.

Like so many events, SMP2021 was an online-only event, while in non-pandemic years the meetings take place in Canberra, allowing participants to get a first-hand impression of the busy schedule of Members of Parliament and their staff.

For 2021, SMP used a format where several 'pre-event' sessions introduced attendees to how government decision-making processes work, and the normal schedule of parliamentarians, ministers and their staff, but also provided tips for communicating science to politicians who are mostly graduates from arts and law degrees and therefore often have no specialist knowledge in STEM subjects. I particularly enjoyed the sessions on 'Conveying STEM work with impact' and 'How to Marie Kondo your writing', while the opening session on 'Understanding the machinery of government' that I had been looking forward to suffered a little bit from the use of specialised terminology.

The program of the main event (15–18 March) was diverse and colourful with presentations from major sponsors, but also a strong focus on ways scientists can influence political decision-making and bring attention to urgent issues. A standout were the sessions with the Superstars of STEM, Indigenous STEM and the National Press Club Address that highlighted obstacles faced by minority groups, the great contributions everyone can make as well as current thoughts on science policy in Australia.

Being trained in how to avoid discipline-specific jargon and still convey a meaningful picture of why my research is important in only 30 s was extremely useful for the meeting with a parliamentarian that is part of the schedule for SMP participants. This meeting with the Member for Eden-Monaro, Ms Kristy McBain was my personal favourite of the event as the discussion not only covered the scientific work of everyone present, but expanded into current science-related and other issues on the political agenda, and March 2021 was packed full of surprising political news.

However, a main focus of SMP is to increase engagement between scientists and the general public, and I particularly noted the strange juxtaposition between the short and hectic electoral cycles that force parliamentarians to focus on issues that will ensure their re-election, versus the frequently stressed fact that it may take some time to bring specific issues to the point where they are being noted.

The key ingredients for making a difference and getting noticed appear to be persistence and excellent preparation. While this may sound obvious, the preparation especially involves more than just preparing a great argument explaining why your idea is important. As part of the preparation one should consider whether other individuals, groups or societies might benefit from a specific idea and might support a pitch. Equally important is getting to know your local member or the minister you will target, although in the latter case the contact may be through the ministerial staff. Ministerial and parliamentary staffers are not a 'second best' – they are actually instrumental in representing portfolios and making sure particular ideas or initiatives are noticed by politicians, and they also tend to stay with particular portfolios in the longer term, which helps when putting forward an idea – repeatedly.

Success is not certain, but chances increase with relevance to the electorate of the person you are talking to, and also with the 'bottomline' – a value proposition that will benefit the portfolio of your contact and/or their constituents.

However, there are other ways to participate that may be more immediately accessible such as participating in submissions to the government as an expert, or engaging with the media so that the issue you are championing is noticed more widely.

There was something new and interesting to learn in each of the SMP sessions I attended, and I enjoyed SMP2021 immensely and would love to experience the event in person in Canberra sometime in the future.

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