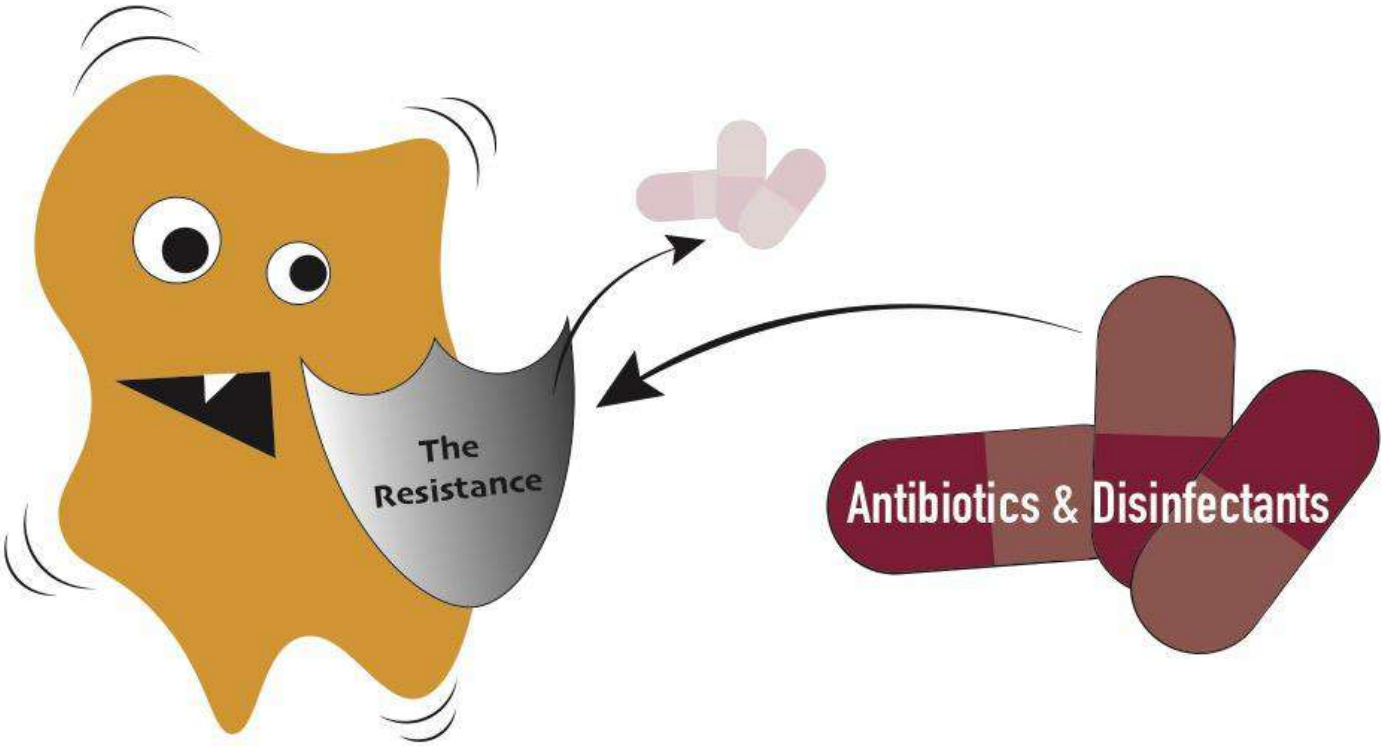
Catalyst

Facets of Biochemistry & Biomedical Sciences

Volume 4 | Issue I | September 2020



Featured Articles:

An Arms Race Against Disinfectant Resistance

Andriana Tetenych[1]
[1]Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Canada

Abstract
Antimicrobials, compounds which inhibit or destroy microorganism growth, have revolutionized modern medicine. However, the overuse and misuse of these antimicrobials has led to the emergence of antibiotic and disinfectant resistance through intrinsic and population resistance mechanisms. Single-cell resistance mechanisms include target alteration, inactivation, efflux pumps, and impermeability, while bacterial populations employ biofilms. New technology, such as quantitative polymerase chain reaction, allows for the quantification of this resistance, which is superior to the minimum inhibitory concentration method. Through quantification, prevention strategies, and further research, full comprehension of combatting disinfectant resistance can be acquired.

Introduction

With exponentially advancing technology and scientific discovery, successful treatment of human pathogens and enhanced clinical outcomes have become prominent in today's society. The discovery of penicillin, in 1928, revolutionized twentieth century medicine; it became the first commercialized antibiotic to fight bacterial infections[1]. However, due to the wide range of microorganism pathogenicity, several types of antimicrobials have been developed. Antimicrobials, which are able to destroy or inhibit microorganism growth, are classified into two main groups (Table 1)[2,3]. Anti-infectives are used to treat infectious diseases and include antibiotics[2]. In contrast, biocides are chemical agents that act outside the human body[3,4]. However, with the wide range of antimicrobial availability, the evolution of resistant bacteria has become a worldwide health concern.

Table 1: Types of antimicrobials. Anti-infectives treat various types of infections in the body. Biocides include treatment of consumer products, inanimate objects, clinical instruments, and surface wounds[2,3,4].

Type	Subtype
Biocides	• Disinfectant • Preservative • Antiseptic
Anti-Infectives	• Antibacterial • Antiviral • Antifungal • Antiparasitic

Antimicrobial Resistance

The widespread use of antimicrobials, especially biocides, has been prevalent for centuries[3,5]. One of the most common antiseptics, alcohol, has been used since ancient civilizations[3,5]. Moreover, the introduction of antibiotics in clinical settings during the 1940's revolutionized modern medicine[3]. However, the overuse and misuse of antibiotics has led to antibiotic resistance through excessively or incorrectly prescribing antibiotics[6,7]. This has made antibiotic resistance one of the most prevalent and costly health concerns in medicine[6,7]. The race to discover novel antibiotics is additionally hindered by regulations such as prolonged approval times placed on pharmaceutical companies and lack of economic motivation[6]. In the United States, antibiotic resistance results in an additional $55 billion per year of excess healthcare costs[8]. Thus, the antibiotic resistance pandemic has prompted the scientific community to extensively study this concern. However, one area with limited research is disinfectant resistance, which will be the focus of this review[9]. Challenges of previous research conducted includes different mechanisms of antimicrobial activity and the lack of specificity of disinfectants[4,9]. Disinfectants, compared to antibiotics, have a wider range of targets and lower antimicrobial activity, which hinders the determination of resistance mechanisms. Thus, by not being able to identify the target of resistance, it poses a challenge to discover novel antimicrobials.

Disinfectant Resistance

Disinfectants are used to kill or hinder microbial growth on inanimate surfaces[2,4]. They are characterized into three main types based on their effectiveness and type of contact: high-level, intermediate, and low-level (Table 2)[10]. High-level disinfectants, which kill microorganisms through liquid immersion, are used in surgical settings on instruments such as endoscopes[10]. Intermediate and low-level disinfectants are used in non-surgical disinfection, such as patient care items, and they kill through liquid contact[10]. Although used in everyday life, the most prevalent disinfectant usage is in healthcare settings[10]. Similar to antibiotics, the misuse of disinfectants such as over-dilution, incorrect duration time, and lack of contact can lead

to disinfectant resistance[10].

Table 2: Types of disinfectants and their targets. High-level disinfectants involve immersion in disinfectant with heat-sensitive items where sterilization is not possible. Intermediate and low-level disinfectants suffice with liquid contact and heat sterilization but cannot kill spores[10].

Type	Targets
High-level	All microorganisms. Exception: high levels of spores.
Intermediate	Microorganisms, most fungi and viruses. Exception: spores.
Low-level	Microorganisms, some fungi and viruses. Exception: spores, mycobacteria.

Disinfectant Resistance Mechanisms

Research on bacterial resistance mechanisms against disinfectants was previously limited due to the broad range of targets and low antimicrobial activity[4]. However, considerable progress has been made in the last 20 years. Resistance mechanisms incorporate multifaceted complexity and are grouped into single-cell or population resistance[11-15]. Single-cell resistance mechanisms involve efflux pumps, impermeability, inactivation, and target alteration[11-16]. These four main mechanisms are considered intrinsic resistance mechanisms[12,14]. In addition, research shows that there are species-specific acquired resistance mechanisms such as bypassing of certain steps in a metabolic pathway[12,16]. However, these are outside the scope of this review.

Single-Cell Resistance

Efflux Pumps

A common way that bacteria acquire disinfectant resistance involves the use of bacterial efflux pumps[16]. These active transporters, located in the cytoplasmic membrane, are responsible for removing toxins from the bacterial cell and contribute to bacterial pathogenicity[16,17]. Encoded by either genomic DNA or DNA from plasmids, there are several types of efflux pumps categorized as multi-drug resistance (MDR) efflux pumps[17]. These include the major facilitator superfamily (MFS), the ATP binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, the multidrug and toxic-compound extrusion (MATE) family, and the resistance nodulation division (RND) family[17]. These efflux pumps are able to export the disinfectants out of the cell (Figure 1A). A notable example involves the qacA pump which is responsible for resistance to chlorhexidine, a disinfectant and antiseptic used to sterilize surgical instruments and patients' skin[18]. In *Staphylococcus aureus,* the qacA efflux pumps use the proton gradient to export chlorhexidine out of the cell, rendering the disinfectant ineffective[12].

Impermeability

Furthermore, impermeability of bacterial membranes to biocides leads to disinfectant resistance (Figure 1B)[14,16]. However, research in this area is controversial. Bacteria are classified into (i) gram-negative bacteria, which possess an outer membrane and a thin layer of peptidoglycan, and (ii) gram-positive bacteria, containing a thicker layer of peptidoglycan[16]. Some research indicates that gram-positive bacteria are less susceptible to biocides due to several factors, such as genetic composition or higher tolerance for oxidizing agents[1,22]. However, most studies conclude that the outer membrane of gram-negative bacteria allows for higher biocide resistance[21,22]. Additionally, this outer membrane can change its hydrophobicity, ultrastructure, protein composition, and fatty acid composition to prevent biocide entry[23-27]. One study showed that chlorhexidine resistant *Pseudomonas aeruginosa* changed their membrane ultrastructure by becoming rough and amorphous. Nonetheless, the mechanisms of changing the outer membrane composition without genetic change are still unknown[16,23-27]. Given the limited research on this topic, such a gap in knowledge may be a possible research direction.

Inactivation

Bacteria can acquire disinfectant resistance through the inactivation of disinfectants (Figure 1C)[14,16]. One prominent example involves the inactivation of Triclosan by *Pseudomonas putida (P. putida)*[28-30]. Triclosan, a common household and medical disinfectant, disables fatty acid synthesis[28-32]. It interacts with the enoyl-acyl carrier protein reductase (ENR) encoded by the *fabI* gene where NAD^+ affinity is increased[31,32]. By forming a NAD^+-Triclosan complex, fatty acid synthesis is unable to occur[31,32]. It was confirmed that *P. putida* resistant bacteria have a homolog to *fabI,* a *fabK* gene, which enables the degradation of Triclosan and its use as a carbon source rather than fatty acid synthesis inhibition[28-32]. Although *P. putida* inactivated Triclosan, this is not a common intrinsic resistance mechanism found in other bacteria [16,28-30]. Overall, more research is necessary to comprehensively understand the degradation mechanism[28-30].

Target Alteration

Most antibiotics have target specificity, so bacterial mutations to modify the target is a common way to confer antibiotic resistance[4,9]. To combat this, disinfectants have a wide range of targets, making it

possible to alter one or a few disinfectant targets through mutations to decrease or eliminate susceptibility (Figure 1D) [11,14,16]. One prominent example involves *Escherichia coli* exhibiting Triclosan resistance[31,32]. Triclosan, as mentioned previously, interacts with the ENR's active site needed for fatty acid biosynthesis[31,32]. Through a missense mutation of *fabI* – the gene encoding the reductase, affinity for Triclosan is decreased and the inactivating NAD^+-Triclosan complex is not formed[31,32]. Thus, fatty acid biosynthesis is able to occur, rendering *E. coli* Triclosan-resistant[31,32].

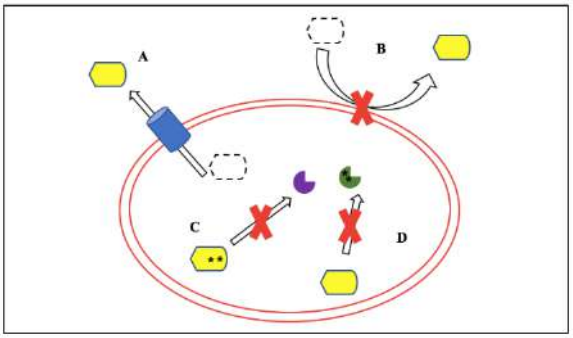


Figure 1: Mechanisms of intrinsic disinfectant resistance. Disinfectant indicated in yellow. **(A)** Disinfectant exported by efflux pump. **(B)** Impermeability to disinfectant. **(C)** Inactivation of disinfectant. **(D)** Target alteration of disinfectant. Alterations indicated by asterisks. Adapted from Poole *et al.* (2002) and Gnanadhas *et al.* (2013)[14,16].

Population Resistance

Biofilms

In addition to the intrinsic resistance mechanisms of bacteria mentioned above, the presence of biofilms enables bacteria to participate in population resistance[11,33]. Biofilms are composed of bacteria surrounded by an extracellular polysaccharide matrix preventing disinfectants from reaching internal layers through steric hindrance (Figure 2A)[33]. A study conducted by Jang *et al*. (2006) showed that chlorine only penetrated 100 µm in a 150-200 µm dairy biofilm[34]. This gives internally placed bacteria a higher chance of survival. Furthermore, electrostatic forces play a role in disinfectant resistance in biofilms[33,35]. Some biofilms may have positively or negatively charged extracellular environment for cell-to-cell communications[33,35]. It was shown that cationic cetylpyridinium chloride, a disinfectant, was hindered when encountering negatively charged biofilms[35]. The attractive forces of the ions prevented the disinfectant from reaching internal layers. In addition to electrostatic and steric forces, bacteria in biofilms are considered heterogeneous due to the different physiological features[33]. Internal bacteria have less access to nutrients and oxygen, accumulating more waste products and thus inducing metabolic modifications[33]. This physiological heterogeneity increases bacterial chances to acquire resistance against disinfectants in biofilms[33]. Overall, many studies confirm the resistance of biofilms to disinfectants mainly through physicochemical interactions rather than steric hindrance[33]. Combined with intrinsic resistance mechanisms of individual bacteria, disinfectant resistance is an ever-growing concern.

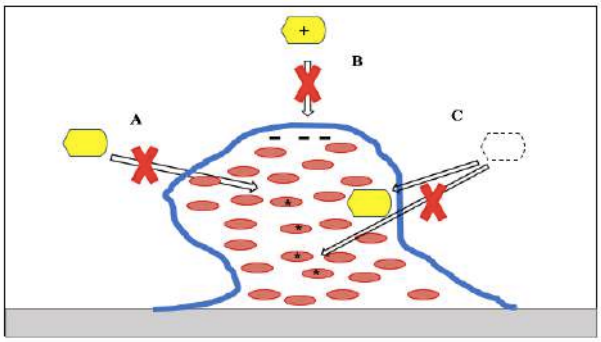


Figure 2: Population resistance using biofilms. Disinfectant indicated in yellow. **(A)** Steric hindrance of disinfectant. **(B)** Hindrance due to electrostatic forces. **(C)** Resistance due to physiological heterogeneity. Modifications indicated by an asterisk. Adapted from Bridier *et al.* (2011) Ganeshnarayan *et al.* (2009) [33-35].

Detection of Disinfectant Resistance

Current: MIC

The most widespread method of disinfectant resistance detection involves calculating the minimum inhibitory concentration (MIC)[36,37]. It is the lowest concentration of disinfectant or antibiotic needed to prevent growth[36]. This efficient and easy method allows for screening of many strains and disinfectants at one time[36,37]. Not only is the MIC used as a diagnostic tool for resistance, but it is also utilized as a measure of activity of disinfectants[37]. For example, the MIC of chlorohexidine diacetate is 1 ug/mL in *S. aureus*. In contrast, an MIC of 0.025 ug/mL is needed for Triclosan in *S. aureus*[38]. However, the MIC is limited to disinfectants that inhibit colony growth rather than destroy. In addition, MIC quantifies the amount of disinfectant needed rather than the bacterial viability of the sample[36].

Future: qPCR

Currently, research methodologies are being developed and perfected to quantify gene expression and bacterial viability in microbial resistance. Quantitative polymerase chain reaction (qPCR) uses florescence and PCR principles to measure amplification signals in thermocyclers[39,40]. By using photo-reactive DNA-binding dye such as propidium monoazide (PMA), the viability of bacterial

cells is able to be detected and quantified through the use of qPCR[39]. When bacterial samples are exposed to disinfectants, cells are lysed due to the dead cells' inability to maintain the cell membrane[39]. PMA binds to the exposed DNA of dead cells and inhibits florescence, which enables quantification between live and dead cells[39]. However, assays vary across bacterial species where no current standardized method exists[39,40].

Cross-Resistance

The alarming, increasing rates of disinfectant and antibiotic resistance increases the potential for cross-resistance to occur and leads to the evolution of superbugs[10,14,16]. Cross-resistance prevails when bacteria resistant to one antimicrobial, such as a disinfectant, become resistant to another antimicrobial[11]. This is seen when both antimicrobials share a target, a pathway, or route of access[11]. Many studies have supported this claim, as seen in Table 3[41-44]. In *P. aeruginosa*, biocide resistance of Triclosan enabled ciprofloxacin resistance through a mutation in the *nfxG* gene[41]. In *Salmonella,* Triclosan facilitated chloramphenicol, erythromycin, imipenem, tetracycline cross-resistance through the increased activity of efflux pumps[42]. The proposed mechanism of this resistance is through an active site mutation enabling these pumps to become MDR and sensitive to other antibiotics[42]. These intrinsic single-cell based resistance mechanisms are promoted by the misuse of disinfectants such as contamination, over-dilution, incorrect duration, and lack of contact[10].

Table 3: Common cross-resistant organisms. Triclosan is a common household and medical disinfectant[31,40-43]. Resistance mechanisms involve intrinsic single-cell based resistance[14,16,40-43].

Organism	Biocide and Antibiotic Resistance	Mechanism
P. aeruginosa	Triclosan, ciprofloxacin	*nfxG* mutation
Mycobacterium	Triclosan, isoniazid	*inhA* mutation
Salmonella	Triclosan, chloramphenicol, erythromycin	Active efflux pumps
S. aureus	Triclosan, ciprofloxacin	Cell membrane alteration

Prevention

In the era of disinfectant and antibiotic resistance, prevention of resistance is not only feasible but should be put into practice in households and hospitals. A crucial step before disinfection involves prior standard cleaning methods, such as removing debris through washing, sterilization if feasible, and following manufacture labels[4,10]. By cleaning surfaces, biofilms and accumulation of other debris that may hinder disinfectant efficacy are removed[4,10]. For example, insufficient bacterial inactivation is the result of failure to properly clean medical instruments[10]. In addition, proper usage of disinfectants should be practiced. Disinfectant resistance develops rapidly through human error mentioned previously such as over dilution, outdated products, and so forth[4]. Following newer, more efficient guidelines and using CDC and FDA-cleared disinfectants will further prevent disinfectant resistance[10].

Conclusion

The ability of bacteria to obtain antibiotic and disinfectant resistance has been a growing concern in modern medicine. The overuse and misuse of these antimicrobials has led to intrinsic and population resistance mechanisms[4,10]. Single-cell resistance mechanisms include target alteration, inactivation, efflux pumps, and impermeability while bacterial populations employ biofilms leading to increased disinfectant resistance[14,16,33]. These strategies allow for the evolution of superbugs which are resistance to many types of antimicrobials through cross-resistance[10,11,14]. Without working antimicrobials against these superbugs, new epidemics may arise in the future[45]. The detection methods of these resistant strains involve calculating the MIC and newer assay developments such as qPCR[36,39]. However, disinfectant resistance is deficient in research in comparison to antibiotic resistance. More research is required to understand the underlying mechanisms[10,16]. In combination with prevention strategies, full comprehension of disinfectant resistance would provide an advantage in this arms race against disinfectant resistance.

Acknowledgments

Special acknowledgments go to Dr. Sara Andres for ongoing support as a supervisor, as well as Dr. Nancy McKenzie and Dr. Felicia Vulcu acting as the thesis support team. This review was completed as part of BIOCHEM 4T15 coursework.

References

1. Fleming, A. Classics in infectious diseases: on the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of B. influenza. *Brit. J. Exp. Path.* **10,** 226-236 (1929).
2. Leekha, S., Terrell, C. L. & Edson, R. S. General principles of antimicrobial therapy. *Mayo Clin. Proc.* **86,** 156-167 (2006).

3. Fraise, A P. Biocide abuse and antimicrobial resistance—a cause for concern? *J. Antimicrob. Chemoth*. **49,** 11–12 (2002).
4. McDonnell, G. & Russell, A, D. Antiseptics and disinfectants: activity, action, and resistance. *Clin. Microbiol. Rev*. **12,** 147-179 (1999).
5. Hajar, R. Alcohol: friend or foe? A historical perspective. *Heart. View.* **1**, 341-344 (2000).
6. Ventola, C. L. The antibiotic resistance crisis: part 1: causes and threats. *Pharm. Therap.* **40**, 277–283 (2015).
7. Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States, 2013. (Centers for Disease Control and Prevention, US Department of Health and Human Services, 2013).
8. Roberts, R. Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. *Clin. Inf. Dis.* **49,** 1175-1184 (2009).
9. Chapman, J. S. Biocide resistance mechanisms. *Int. Biodeter. Biodegr.* **51,** 133-138 (2003).
10. Weber, D. J., Rutala, W. A., & Sickbert-Bennett, E. E. Outbreaks associated with contaminated antiseptics and disinfectants. *Antimicrob. Agents.Ch.* **51,** 4217-4224 (2007).
11. Chapman, J. S. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeter. Biodegr.* **51,** 271-276 (2003).
12. Russell, A. Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *J. Appl. Microbiol*. **92,** 121-135 (2002).
13. Cloete, T. E. Resistance mechanisms of bacteria to antimicrobial compounds. *Int. Biodeter. Biodegr*. **51,** 277-282 (2003).
14. Poole, K. Mechanisms of bacterial biocide and antibiotic resistance *J. Appl. Microbiol*. **92,** 55-64 (2002).
15. Russell, A. D. Similarities and differences in the responses of microorganisms to biocides, *J. Antimicrob. Chemoth*. ***52,*** 750-763 (2003).
16. Gnanadhas, D. P., Marathe, S. A. & Chakravortty D. Biocides – resistance, cross-resistance mechanisms and assessment. *Expert Opin. Inv. Drug*. **22,** 191-206 (2013).
17. Piddock, L. J. Multidrug-resistance efflux pumps? not just for resistance. *Nat. Rev. Microbiol*. **4,** 629 (2006).
18. Adams, D., Quayum, M., Worthington, T., Lambert, P. & Elliott, T. Evaluation of a 2% chlorhexidine gluconate in 70% isopropyl alcohol skin disinfectant. *J. Hosp. Infect.* **61,** 287-290 (2005).
19. Littlejohn, T. G., Paulsen, I. T., Gillespie, M. T., Tennent, J. M., Midgley, M., Jones, I. G., Purewal, A.S. & Skurray, R. A. Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. *FEMS. Microbiol. Lett.* **74,** 259– 265 (1992).
20. Dance, D. A., Pearson, A. D., Seal, D. V. & Lowes, J. A. A hospital outbreak caused by a chlorhexidine and antibiotic-resistant *Proteus mirabilis*. *J. Hosp. Infect.* **10,** 10-16 (1987).
21. Bridier, A., Briandet, R., Thomas, V. & Dubois-Brissonnet. F. Comparative biocidal activity of peracetic acid, benzalkonium chloride and ortho-phthalaldehyde on 77 bacterial strains. *J. Hosp. Infect.* **78,** 208-213 (2011).
22. Murtough, S. M., Hiom, S. J., Palmer, M. & Russell, A. D. Biocide rotation in the healthcare setting: is there a case for policy implementation? *J. Hosp. Infect.* **48,** 1-6 (2001).
23. Tattawasart, U., Maillard, J.-Y., Furr, J.R. & Russell, A.D. Development of resistance tochlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri and* changes in antibiotic susceptibility. *J. Hosp. Infect.* **42,** 219-229 (1999).
24. Tattawasart, U., Hann, A. C., Maillard, J. Y., Furr, J. R. & Russell, A. D. Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*. *J. Antimicrob. Chemoth.* **45,** 145-152 (2000).
25. Gandhi, P. A., Sawant, A. D., Wilson, L.A. & Ahearn, D.G. Adaptation and growth of *Serratia marcescens* in contact lens disinfectant solutions containing chlorhexidine gluconate. *Appl. Environ. Microb.* **59,** 183-188 (1993).
26. Guerin-Mechin, L., Dubois-Brissonnet, F., Heyd, B. & Leveau, J. Y. Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. *J. Appl. Microbiol.* **87,** 735-742 (1999).
27. Guerin-Mechin, L., Dubois-Brissonnet, F., Heyd, B. & Leveau, J. Y. Quaternary ammonium compound stresses induce specific variations in fatty acid composition of *Pseudomonas aeruginosa*. *Int. J. Food. Microbiol.* **55,** 157-159 (2000).
28. Van Klingeren, B. & Pullen, W. Glutaraldehyde resistant mycobacteria from endoscope washers. *J. Hosp. Infect.* **25,** 147-149 (1993).

29. Hay, A. G. & Dees, P. M.; Sayler, G. S. Growth of a bacterial consortium on triclosan. *FEMS. Microbiol. Ecol.* **36,** 105-112 (2001).
30. Meade, M. J., Waddell, R. L. & Callahan, T. M. Soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. denitrificans inactivate triclosan in liquid and solid substrates. *FEMS. Microbiol. Lett.* **204,** 45-48 (2004).
31. Heath, R. J., Yu, Y. T., Shapiro, M. A., Olson, E. & Rock, C.O. Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis. *J. Biol. Chem.* **273,** 30316– 30320 (1998).
32. Heath, R. J., Rubin, J. R., Holland, D. R., Zhang, E., Snow, M. E. & Rock, C. O. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *J. Biol. Chem.* **274,** 11110–11114 (1999).
33. Bridier, A., Briandet, R., Thomas, V. & Dubois-Brissonnet, F. Resistance of bacterial biofilms to disinfectants: a review. *Biofouling*. **27,** 1017-1032 (2011).
34. Jang, A., Szabo, J., Hosni, A. A., Coughlin, M. & Bishop, P. L. Measurement of chlorine dioxide penetration in dairy process pipe biofilms during disinfection. *Appl. Microbiol. Biotechnol*. **72,** 368–376 (2006).
35. Ganeshnarayan, K., Shah, S. M., Libera, M. R., Santostefano, A. & Kaplan, J. B. Poly-N-acetylglucosamine matrix polysaccharide impedes fluid convection and transport of the cationic surfactant cetylpyridinium chloride through bacterial biofilms. *Appl. Environ. Microbiol*. **75,** 1308–1314 (2009).
36. Langsrud, S., Sidhu, M. S., Heir, E. & Holck, A. L. Bacterial disinfectant resistance—a challenge for the food industry. *Int. Biodeter. Biodegr.* **51,** 283-290 (2003).
37. Andrews, J. M. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemoth.* **48,** 5-16 (2001).
38. Suller, M. T. & Russell, A. D. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcus. *J. Hosp. Infect.* **43,** 281-291 (1999).
39. Nocker, A., Cheung, C. Y. & Camper, A. K. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Meth*. **67,** 310-320 (2006).
40. Bustin, S. A., Beaulieu, J. F., Huggett, J., Jaggi, R., Kibenge, F. S., Olsvik, P. A. et al. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC. Mol. Biol.* **11,** 74 (2010).
41. Chuanchuen, R., Beinlich, K. & Hoang, T. T. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. *Antimicrob. Agents. Ch.* **45,** 428-432 (2001).
42. Braoudaki, M. & Hilton, A. C. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. *J. Clin. Microbiol*. **42,** 73-78 (2004).
43. Slayden, R. A., Lee, R. E. & Barry, C. E. Isoniazid affects multiple components of the type II fatty acid synthase system of Mycobacterium tuberculosis. *Mol. Microbiol*. **38,** 514-525 (2000).
44. Tkachenko, O., Shepard, J. & Aris, V. M. A triclosan-ciprofloxacin cross-resistant mutant strain of Staphylococcus aureus displays an alteration in the expression of several cell membrane structural and functional genes. *Res. Microbiol.* **158,** 651-658 (2007).
45. Collingnon, P., & Beggs, J J. Socioeconomic Enablers for Contagion: Factors Impelling the Antimicrobial Resistance Epidemic. *Antibiot*. **8**, 86 (2019).

Probiotics and Obesity: The Therapeutic Potential of *Lactobacillus gasseri* SBT2055

Samina Idrees[1]
[1]Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Canada

Abstract
Obesity is an ever-growing problem in today's world. This disease serves as a significant public health burden in Canada, costing billions of dollars annually. However, more troubling is the impact this disease has on individual health and mortality. Diet and exercise have fallen short at combatting the growing prevalence of obesity, emphasizing the need for alternative treatment options. Recent research highlights the potential therapeutic effect of probiotics. Specifically, there is an abundance of research supporting the anti-obesity effects of the probiotic *Lactobacillus gasseri* SBT2055 (LG2055). Here, we aimed to critically evaluate two of the studies supporting these findings. We conclude that LG2055 holds therapeutic potential for obesity, however more research is needed to substantiate this claim.

Introduction

Obesity is a metabolic disorder which has recently begun to plague the lives of millions around the globe. Specifically, in Canada, the percentage of adults with obesity has more than doubled since 1978-1979[1]. Currently, 64% of Canadian adults and about 14% of Canadian youth are obese[2, 3]. This is concerning because obesity is associated with an increased risk of type 2 diabetes, heart disease, certain cancers and premature death[4, 3]. It is estimated that excess weight causes 1 in 10 premature deaths among Canadians between 20 and 64 years of age[3]. Additionally, obesity costs Canada around $11.1 billion annually – accounting for both direct healthcare costs which are estimated to be between $4.6 to 7.1 billion and indirect impacts (i.e. loss of productivity)[3,5]. Considering the significant burden this disease puts on health and economy, finding an effective cure becomes of paramount importance.

The multifactorial and complex nature of obesity makes it difficult to treat. Obesity often develops as a result of a sustained positive energy imbalance which can be caused by an increase in energy intake or a decrease in energy expenditure (i.e. decreased activity levels)[6]. The excess energy leads to weight gain and excess adiposity which is likely a result of increased lipid absorption at the level of the small intestine[7]. The cornerstone of obesity treatment has long been lifestyle modification (i.e. exercise and diet), however these methods have been unable to combat the rapid spread of this disease[8]. A growing body of evidence suggests that the biological adaptations associated with obesity persist through remission, preventing patients from ever truly recovering (e.g. neuronal changes which cause individuals to feel a reward deficit while dieting, thereby promoting overeating)[8]. Behaviorally induced weight loss strategies are therefore not sufficient for the treatment of this disorder – putting an emphasis on the need for more biologically based interventions[8]. Several biological based treatment routes have been proposed, however more research is needed to evaluate their efficacy.

One proposed treatment for obesity involves the administration of probiotics or prebiotics aimed at altering the composition of the gut microbiota. The gut microbiota has been implicated in obesity as it is an important player in metabolism (i.e. digestion and absorption of nutrients) and immune system function. Research findings indicate that there are obesity-associated changes in the gut microbiota which allow for an increase in energy absorption[9]. Additionally, evidence suggests that this trait is transferable as transplanting obese mouse microbiota leads to obesity in germ-free mice[9]. In light of this information, there is a reasonable basis to target the gut microbiota in obesity treatment.

If used in conjunction with changes in diet and exercise, the administration of probiotics or prebiotics may provide a more effective method for obesity treatment. A recent finding by Scheiman *et al*. (2019) helps push this narrative as they demonstrated the ability of probiotic intervention to effectively boost exercise capacity in mice[10]. Probiotics are living microorganisms, commonly referred to as the 'good' bacteria (i.e. those involved in conferring health benefits to the host). Ingestion of probiotics can transmit positive health effects if given in adequate quantities[11]. Specifically, probiotics have been shown to improve lactose intolerance, prevent diarrhea, and have anti-tumour and anti-obesity effects[12-15]. Alternatively, prebiotics refer to indigestible parts of food such as fibre which is not digested by the gut and instead enters the colon to

be fermented by the bacteria there. Prebiotics serve as a food source for the good bacteria within the gut, and they have been shown to reduce hypercholesteremia[16] and have anti-obesity effects[17]. Both can be ingested as part of common foods (e.g. yogurt) or incorporated into supplements. In short, the ingestion of probiotics or prebiotics can have numerous beneficial effects for host metabolic health.

One probiotic showing promise as a potential therapeutic for obesity is *Lactobacillus gasseri* SBT2055 (LG2055). LG2055 is thought to colonize, proliferate and subsequently inhibit lipid absorption at the level of the small intestine[18, 15]. Ogawa *et al*. (2015) found LG2055 supplementation to be associated with increased lipid levels in human feces, indicating decreased lipid absorption[19]. Decreased lipid absorption as a result of LG2055 likely occurs through suppression of pancreatic lipase-mediated fat hydrolysis[19]. The proposed mechanism of action does not involve direct inhibition of the lipase, instead LG2055 likely plays a role in increasing fat emulsion size which is linked to decreased specific surface area. Consequently, the lipase has less area on which it can act – leading to suppression of its activity[19]. Through this mechanism, LG2055 has the capacity to alter the energy balance ratio by decreasing fat absorption and increasing energy output – positioning it as a prime candidate for obesity treatment.

LG2055 also has anti-inflammatory properties which could help combat the chronic-low grade inflammation that often presents itself alongside obesity[20]. Targeting this inflammation is central to helping improve the prognosis of obesity as chronic low-grade inflammation is a known cause of insulin resistance, a major risk factor for type 2 diabetes[21]. Kawano *et al*. (2016) highlighted LG2055's ability to attenuate several key hallmarks of obesity-related inflammation including high-fat diet (HFD)-mediated increases in intestinal permeability and adipose tissue inflammation[22]. Additionally, Miyoshi *et al*. (2014) provided evidence for a link between LG2055 treatment and decreased expression of pro-inflammatory genes in the adipose tissue of obese mice[20]. Hence, the anti-inflammatory properties of LG2055 provide another avenue for its anti-obesity effects.

Although inflammation is a key player in obesity, the increase in number or enlargement of adipocytes are also important features of this disease[23]. Sato *et al*. (2008) linked LG2055 supplementation to a decrease in adipocyte size after reporting a significant reduction in the size of mesenteric and retroperitoneal adipocytes in LG2055-treated rats fed an HFD[24]. Considering the close relationship between adipocyte size and excess fat mass, this finding provides support for LG2055 as a candidate for obesity treatment. Additionally, many studies have reported decreased liver triglyceride (TG) levels in animal models post-LG2055 treatment, thereby offering further support for LG2055 as a therapeutic for obesity[20,24].

LG2055 was initially isolated from human feces and it is part of the larger group of lactic acid bacteria[25]. It is often incorporated into dairy products where it functions to increase bio-preservation and digestibility[25]. This probiotic can also be found in dietary supplements as its consumption has been associated with several beneficial health effects. Such effects of LG2055 consumption are further enhanced by its safety profile. *Lactobacillus gasseri* strains are considered safe, but they may cause some unwanted side-effects such as gas or bloating. Nonetheless, their therapeutic potential seems to outweigh these minor forms of discomfort. Along with this low risk factor, proposing LG2055 as a therapeutic for obesity is also feasible as this probiotic is already widely available in capsule form. However, it is important to note that this probiotic would not be recommended to everyone – namely those with compromised immune systems or those taking immunosuppressive medications as they are more prone to infection of cardiac tissue[26].

Both animal studies and human trials have demonstrated the ability of LG2055 to alleviate obesity symptoms. The purpose of this report is to critique these studies in order to take an informed stance on the suitability of LG2055 as a treatment for obesity.

Critical Review

Many studies have reported on the anti-obesity effects of LG2055. A study by Kadooka *et al*. (2013) suggests that LG2055 reduces abdominal adiposity and that constant intake of this probiotic may be needed to maintain its anti-obesity effects[27]. The study found LG2055 intake to be associated with lowered abdominal adiposity in individuals with obese-like tendencies. They conducted a randomised, controlled trial involving 210 Japanese adults who had large visceral fat stores but were otherwise considered healthy. The individuals in the study were randomly divided into three groups: those who received fermented milk (FM) containing 10^7 CFU of LG2055/g; those who received FM containing 10^6 CFU of LG2055/g; or a control group

Table 1. Percentage changes and corresponding measures from baseline in abdominal fat areas. Table modified from Kadooka *et al.* (2013)[27]. (Mean values and 95% confidence intervals)

Parameters	Group	Week 8 Mean	(95% CI)	Week 12 Mean	(95% CI)
Visceral‡	10^7 dose				
	%	– 4.8**	– 7.6, – 1.9	– 8.5**†	– 11.9, – 5.1
	cm^2	– 5.3	– 8.4, – 2.2	– 9.6	– 14.3, – 4.9
	10^6 dose				
	%	– 5.6**	– 8.1, – 3.1	– 8.2**†	– 10.8, – 5.7
	cm^2	– 5.8	– 8.3, – 3.2	– 8.6	– 11.3, – 5.9
	Control				
	%	– 1.2	– 4.1, 1.7	– 0.7	– 3.8, 2.5
	cm^2	– 1.3	– 4.5, 2.0	– 0.4	– 4.1, 3.2

Mean values were significantly different for within-group comparisons from baseline: * $P<0.05$, ** $P<0.01$.
†Mean values were significantly different for between-group comparisons from control ($P<0.05$).
‡ There was a significant group × time interaction effect ($P<0.05$).

who received FM containing 0 CFU of LG2055/g. The participants consumed 200 g of FM per day for 12 weeks. By the end of the 12 weeks, the researchers observed a decrease in visceral abdominal fat area in both the 10^7 and 10^6 groups (Table 1). Additionally, individuals in the 10^7 and 10^6 groups had reductions in BMI, waist and hip circumference, and body fat mass (Table 2). The researchers also found that four weeks after ending the treatment, these effects were reduced (Table 2).

This study bears a near-perfect resemblance to a previous report on the effect of FM containing 10^8 CFU of LG2055/g on individuals with obese-like tendencies; the fundamental difference is in the administered dose of LG2055. All other variables remained nearly constant, including the formation of the FM, the study schedule and protocol[27,28]. The calibre of participants was also similar as both trials recruited healthy Japanese males with large areas of visceral fat[27,28]. This approach of changing one variable from a previous experiment and keeping all else constant is an effective way to extrapolate accurate information. As a result of employing this approach, the researchers were able to indicate that even at lower doses of LG2055 (at the recommended minimum and intermediate levels), the probiotic was equally potent in its ability to reduce abdominal visceral fat area[27]. These results have an added layer of credibility due to the authors' strict adherence to the methods used by the previous study, however this characteristic may also serve to undermine the generalisability of this research.

The participants within this study were of a specific cohort, and so the results may not be extrapolative of the wider population. Specifically, all participants were of Japanese descent and their average age was around 47 years[27]. This is troublesome because the population demographics of those who could benefit from this probiotic are variable in terms of age and race. This study aims to describe LG2055 as an effective way to lower abdominal adiposity in adults, however this conclusion is based on results from a specific subsection of the population. Future studies should include individuals from a wide range of backgrounds and ages, as this would better support the view of LG2055 as a probiotic capable of lowering abdominal adiposity in humans.

Additionally, the authors made an apparent generalisation in the discussion when mentioning the trial's ability to demonstrate LG2055's anti-obesity effects. The central issue traces back to the fact that the subjects within the study were not obese[27]. The subjects were healthy despite having large visceral fat stores. Therefore, although the participants had obese-like tendencies, they did not serve as a true model for human obesity. A more accurate statement would be to say that LG2055 helped reverse abdominal adiposity in healthy individuals, however more research is needed to confirm the same effect in obese patients.

Furthermore, the study did not control for diet, as participants were allowed to continue with their normal eating habits[27]. The over-arching influence of diet on the composition of the gut microbiota has been widely reported[29,30]. Therefore, it is difficult to accept the results from this study as they suggest a direct link between LG2055 treatment and reduced abdominal adiposity, without accounting for variability in participants' diets. Future studies should include more strict diet restrictions or record daily consumption habits as this would more accurately elucidate LG2055's effect on obesity symptoms.

Table 2. Percentage changes and corresponding measures from baseline in BMI, waist, hip and fat mass in 10^7, 10^6 and control mice. Table modified from Kadooka *et al.* (2013)[27].

(Mean values and 95% confidence intervals)

		Week 8		Week 12		4 weeks after finishing consumption	
Parameters	Group	Mean	95% CI	Mean	95% CI	Mean	95% CI
BMI ‡‡	10^7 dose						
	%	– 0.6**†	– 1.0, – 0.2	– 1.1**††	– 1.6, – 0.6	– 0.5	– 0.9, 0.0
	kg/m²	– 0.2	– 0.3, – 0.1	– 0.3	– 0.4, – 0.2	– 0.1	– 0.3, 0.0
	10^6 dose						
	%	– 1.0**††	– 1.4, – 0.6	– 1.6**††	– 2.1, – 1.1	– 0.6*†	– 1.0, – 0.1
	kg/m²	– 0.3	– 0.4, – 0.2	– 0.4	– 0.5, – 0.3	– 0.2	– 0.3, 0.0
	Control						
	%	0.3	0.0, 0.6	0.3	– 0.1, 0.7	0.4	0.0, 0.8
	kg/m²	0.1	0.0, 0.2	0.1	– 0.1, 0.2	0.1	0.0, 0.3
Waist ‡‡	10^7 dose						
	%	– 0.9**†	– 1.3, – 0.6	– 1.4**††	– 1.8, – 1.0	– 0.8**	– 1.3, – 0.4
	cm	– 0.9	– 1.2, – 0.5	– 1.3	– 1.7, – 0.9	– 0.8	– 1.2, – 0.4
	10^6 dose						
	%	– 0.8**	– 1.2, – 0.4	– 1.2**††	– 1.7, – 0.7	– 0.7**	– 1.2, – 0.2
	cm	– 0.7	– 1.1, – 0.4	– 1.1	– 1.5, – 0.7	– 0.7	– 1.1, – 0.3
	Control						
	%	0.0	– 0.4, 0.3	– 0.1	– 0.4, 0.3	– 0.2	– 0.6, 0.2
	cm	– 0.1	– 0.4, 0.3	– 0.1	– 0.5, 0.3	– 0.2	– 0.7, 0.3
Hip ‡‡	10^7 dose						
	%	– 0.8**†	– 1.0, – 0.5	– 1.2**††	– 1.5, – 0.9	– 0.5**	– 0.8, – 0.2
	cm	– 0.8	– 1.0, – 0.5	– 1.2	– 1.5, – 0.9	– 0.5	– 0.8, – 0.2
	10^6 dose						
	%	– 0.5**	– 0.8, – 0.3	– 0.9**††	– 1.1, – 0.6	– 0.3*	– 0.6, – 0.1
	cm	– 0.6	– 0.8, – 0.3	– 0.9	– 1.1, – 0.6	– 0.4	– 0.6, – 0.1
	Control						
	%	– 0.2	– 0.4, 0.0	– 0.2	– 0.4, 0.1	– 0.2	– 0.4, 0.1
	cm	– 0.2	– 0.5, 0.1	– 0.2	– 0.5, 0.1	– 0.2	– 0.5, 0.2
Fat mass ‡‡	Value (%)	0.4	0.1, 0.6	0.6	0.3, 0.9	0.9	0.5, 1.2
	10^7 dose						
	%	– 0.9†	– 2.1, 0.4	– 2.4**††	– 3.8, – 0.9	– 0.2††	– 1.6, 1.2
	kg	– 0.3	– 0.6, 0.0	– 0.6	– 1.0, – 0.3	– 0.1	– 0.4, 0.2
	10^6 dose						
	%	– 0.8†	– 1.9, 0.2	– 2.2**†††	– 3.4, – 1.0	– 0.3††	– 1.5, 0.8
	kg	– 0.2	– 0.4, 0.0	– 0.5	– 0.7, – 0.2	0.0	– 0.3, 0.2
	Control						
	%	1.5**	0.7, 2.3	2.2**	1.2, 3.3	3.3**	2.2, 4.5
	kg	0.4	0.1, 0.6	0.5	0.2, 0.8	0.7	0.4, 1.1

Mean values were significantly different for within-group comparisons from baseline: * $P<0.05$, ** $P<0.01$.
Mean values were significantly different for between-group comparisons from control: †$P<0.05$, ††$P<0.01$.
‡‡ There was a significant group × time interaction effect ($P<0.01$)

A study by Miyoshi *et al*. (2014) also highlights LG2055's ability to attenuate obesity-related symptoms[20]. Researchers observed a relationship between LG2055 treatment, and decreased weight and adipose tissue mass (retroperitoneal/perineal and epididymal) in diet-induced obese mice[20]. The mice were divided into three groups: 5% fat group (5% fat); 10% fat group (10% fat); and 10% fat group with LG2055 (10% fat-LG). The group names indicate the proportion of fat the mice received from their diet. The mice were fed these diets for a period of 24 weeks in order to induce obesity. At the end of the 24 weeks, the mice were anesthetized, and body weight, adipose tissue mass, liver TG levels, expression of inflammatory genes in fat tissue and the expression of lipogenic and lipolytic genes in the liver were quantified. The researchers found LG2055 intake to be associated with decreased liver TG levels (Table 3), decreased expression of pro-inflammatory genes (e.g. CCL2) in epididymal adipose tissue (Table 4), and decreased expression of lipogenic genes (e.g. ACC1) in the liver (Table 5).

A distinct feature of this study centres around the route of administration for LG2055. Miyoshi *et al*. (2014) did not use FM containing LG2055 as the delivery method for this probiotic[20]. Previously, many studies have reported anti-obesity effects in animal models upon administration of FM containing LG2055[15, 24]. This study suggests that LG2055 alone is the source of these effects – not the metabolites present in FM[20]. This has implications for determining the most effective delivery route for this probiotic.

Although the route of administration provides more validity to the results, the method used to create obese mice does not resemble the development of obesity in humans. The researchers fed the mice a 5% or 10% fat diet for 24 weeks in order to induce obesity. However, it could be more valuable to induce obesity through administration of an HFD (i.e. 60% fat, 20% protein and 20% carbohydrates) as this would allow for a model of obesity that better resembles the human condition[31]. The results from such an experiment would provide more insight on the potential therapeutic role of LG2055 in human obesity.

Moreover, having a group of mice on a normal chow diet (NCD) could serve as a useful addition to this study. This trial included mice on a 5% or 10% fat diet. An NCD and a low-fat diet (e.g. 5% fat) are not equivalent as they elicit different phenotypes in mice[22]. For example, mice on a low-fat diet have significantly higher total serum cholesterol levels than NCD mice[32]. Obesity is often characterised by high LDL cholesterol levels in the blood, and so a low-fat diet would not serve as an accurate depiction of healthy mouse physiology[33]. Therefore, the researchers failed to demonstrate the effects of LG2055 on healthy mice when doing so could allow for post-treatment comparisons between healthy and obese mice. This in turn, could allow us to make inferences on whether LG2055 serves a preventative role against obesity in healthy individuals.

Table 3. Morphometric and biochemical parameters between 5% fat, 10% fat and 10% fat-LG mice. Table modified from Miyoshi *et al*. (2014)[20].

Parameters	Experimental groups		
	5% fat (*n* = 10)	10% fat (*n* = 9)	10% fat-LG (*n* = 10)
Average diet intake (g/day)	3.62 ± 0.17	3.78 ± 0.26	3.71 ± 0.29
Average energy intake (kJ/day)	58.28 ± 2.73^{a}	64.64 ± 4.47^{b}	63.44 ± 5.00^{b}
Initial body weight (g)	21.78 ± 1.24	21.79 ± 1.01	21.55 ± 1.45
Final body weight (g)	31.41 ± 2.25^{a}	39.15 ± 2.18^{b}	35.48 ± 2.26^{c}
Adipose tissue weight (% of final body weight)			
Mesenteric	2.24 ± 0.27^{a}	3.10 ± 0.39^{b}	2.69 ± 0.40^{b}
Epididymal	3.14 ± 1.14^{a}	5.78 ± 0.55^{b}	4.63 ± 1.10^{c}
Perirenal/Retroperitoneal	1.22 ± 0.46^{a}	2.47 ± 0.31^{b}	1.89 ± 0.43^{c}
Total	6.60 ± 1.75^{a}	11.34 ± 0.90^{b}	9.21 ± 1.80^{c}
Liver TG (mg/g tissue weight)	14.92 ± 8.52^{a}	47.18 ± 20.11^{b}	30.39 ± 20.32^{ab}

Each value represents the mean ± SD.
Values with different superscript letters (a–c) within the same row differ significantly from each other ($P<0.05$, Games–Howell test).

Table 4. Expression of pro-inflammatory genes in epididymal adipose tissue. Table modified from Miyoshi *et al*. (2014)[20].

	5% fat	10% fat	10% fat-LG
Pro-inflammatory genes			
CCL2	1.00	1.87	1.20
CCR2	1.00	1.40	0.65
ICAM1	1.00	1.59	1.55
IL6		ND	
RBP4	1.00	0.69	0.88
RETN	1.00	0.59	0.84
PAI-1	1.00	1.41	1.06
TNFα		ND	

Data are represented as a fold change relative to levels in the 5 % fat group.
ND not determined.

Table 5. Expression of lipogenic genes in liver. Table modified from Miyoshi *et al*. (2014)[20].

	5% fat`	10% fat	10% fat-LG
Lipogenic genes			
ACC1	1.00	1.24	1.03
FAS	1.00	1.36	0.90
SREBP1	1.00	1.24	0.94

Data are represented as a fold change relative to levels in the 5 % fat group.

Conclusion

In summary, LG2055 shows promise as a treatment for obesity. The anti-obesity effects of LG2055 have been widely reported. Kadooka *et al*. (2013) showed that LG2055 was effective in attenuating several obesity-related symptoms in humans[27]. The researchers associated LG2055 intake (at the recommended minimum or intermediate level) to decreased visceral abdominal fat area (Table 1), reductions in BMI, waist and hip circumference, and body fat mass (Table 2)[27]. However, it is difficult to make generalisations from these data as they focused on a specific subsection of the population (Japanese adults around 47 years of age) who were not obese. Nonetheless, the results from this study are strengthened through the employed methodology. The methods used in this study were nearly identical to those from a previous study[27,28], the only difference being in the administered dose of LG2055. This allows for less variability in the results, while also validating the anti-obesity effects of LG2055 observed in the previous study[27,28].

In addition, a study by Miyoshi *et al*. (2014) advanced a similar hypothesis as they linked LG2055 treatment with decreased weight and adipose tissue mass in diet-induced obese mice[20]. The researchers found LG2055 intake to be associated with decreased liver TG levels (Table 3), decreased expression of pro-inflammatory genes in epididymal adipose tissue (Table 4) and decreased expression of lipogenic genes in the liver (Table 5). The diet-induced obese mice used within this study were not ideal for modelling obesity in humans. However, the results from this study provide essential information in relation to LG2055's ability to attenuate obesity-related symptoms[20]. The data also indicates that the consumption of LG2055 alone is sufficient to elicit its anti-obesity effects.

These data highlight LG2055's ability to improve obesity symptoms, further promoting it as a potential therapeutic for obesity. LG2055 reduced visceral abdominal fat area and fat mass in humans[27], and it caused a decrease in adipose tissue mass in mice[20]. These events indicate a shift away from the obese phenotype and they are likely caused by LG2055's ability to decrease fat absorption at the level of the small intestine through suppressing pancreatic-lipase mediated fat hydrolysis[19]. This leads to an increase in energy output and a decrease in energy intake, thereby working to help rescue the sustained positive energy imbalance in obesity[6]. In light of this knowledge, LG2055 presents itself as a prime

candidate for obesity treatment, however more research should be done to determine its effectiveness, particularly in conjunction with behaviorally induced weight loss strategies.

Acknowledgements

I would like to thank Patrycja Jazwiec, a PhD candidate in the Sloboda Lab, for her support and guidance through this project. This work was completed as part of the requirements for BIOCHEM 4M03, Winter 2019, at McMaster University.

References

1. Public Health Agency of Canada. Obesity in Canadian adults: it's about more than just weight. https://health-infobase.canada.ca/datalab/adult-obesity-blog.html (2017).
2. Public Health Agency of Canada. Tackling Obesity in Canada: Obesity and Excess Weight Rates in Canadian Adults.https://www.canada.ca/en/public-health/services/publications/healthy-living/obesity-excess-weight-rates-canadian-adults.html (2018).
3. The Standing Senate Committee on Social Affairs, Science and Technology Senate. Obesity in Canada. https://sencanada.ca/content/sen/committee/421/SOCI/Reports/2016-02-25_Revised_report_Obesity_in_Canada_e.pdf (2016).
4. Obesity Canada. Obesity in Canada. https://obesitycanada.ca/obesity-in-canada/ (accessed, 15 April 2019).
5. Canadian Obesity Network. Report Card on Access to Obesity Treatment for Adults in Canada 2017. https://obesitycanada.ca/wp-content/uploads/2017/07/Report-Card-Full-Version-PDF.pdf (2017).
6. Romieu, I. et al. Energy balance and obesity: what are the main drivers? *Cancer. Cause. Control.* **28**, 247-258 (2017).
7. Martinez-Guryn, K. et al. Small intestine microbiota regulate host digestive and absorptive adaptive responses to dietary lipids. *Cell. Host. Microbe.* **23**, 458-469 (2018).
8. Ochner, C. N., Tsai, A. G., Kushner, R. F. & Wadden, T. A. Treating obesity seriously: when recommendations for lifestyle change confront biological adaptations. *Lancet. Diabetes. Endo.* **3**, 232-234 (2015).
9. Turnbaugh, P., Ley, R., Mahowald, M. et al. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444,** 1027–1031 (2006).
10. Scheiman, J., Luber, J.M., Chavkin, T.A. *et al.* Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat. Med.* **25,** 1104–1109 (2019). https://doi.org/10.1038/s41591-019-0485-4
11. Araya, M. et al. Guidelines for the evaluation of probiotics in food. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (2002).
12. Vonk, R. J., Reckman, G. A., Harmsen, H. J. & Priebe, M. G. Probiotics and lactose intolerance. *Probiotics:IntechOpen* (2012).
13. Szymański, H et al. Treatment of acute infectious diarrhoea in infants and children with a mixture of three Lactobacillus rhamnosus strains–a randomized, double-blind, placebo-controlled trial. *Aliment. Pharm. Ther.* **23**, 247-253 (2006).
14. Lee, J. H. et al. The production of surfactin during the fermentation of cheonggukjang by potential probiotic Bacillus subtilis CSY191 and the resultant growth suppression of MCF-7 human breast cancer cells. *Food. Chem.* **131**, 1347-1354 (2012).
15. Hamad, E. M. et al. Milk fermented by Lactobacillus gasseri SBT2055 influences adipocyte size via inhibition of dietary fat absorption in Zucker rats. *Br. J. Nutr.* **101**, 716–724 (2009).
16. Parnell, J. A. & Reimer, R. A. Effect of prebiotic fibre supplementation on hepatic gene expression and serum lipids: a dose–response study in JCR: LA-cp rats. *Br. J. Nutrs.* **103**, 1577-1584 (2010).
17. Nicolucci, A. C. et al. Prebiotics reduce body fat and alter intestinal microbiota in children who are overweight or with obesity. *Gastroenterology* **153**, 711-722 (2017).
18. Fujiwara, S., Seto, Y., Kimura, A. & Hashiba, H. Establishment of orally-administered *Lactobacillus gasseri* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *J. Appl. Microbiol.* **90**, 343-352 (2001).
19. Ogawa, A., Kobayashi, T., Sakai, F., Kadooka, Y. & Kawasaki, Y. Lactobacillus gasseri SBT2055 suppresses fatty acid release through enlargement of fat emulsion size in vitro and promotes fecal fat excretion in healthy Japanese subjects. *Lipids. Health. Dis.* **14**, 20 (2015).
20. Miyoshi, M., Ogawa, A., Higurashi, S. & Kadooka, Y. Anti-obesity effect of Lactobacillus gasseri SBT2055 accompanied by inhibition of pro-inflammatory gene expression in the visceral adipose tissue in diet-induced obese mice. *Eur. J. Nutr.* **53**, 599–606 (2014).

21. Osborn, O. & Olefsky, J. The cellular and signaling networks linking the immune system and metabolism in disease. *Nat. Med.* **18,** 363–374 (2012).
22. Kawano, M., Miyoshi, M., Ogawa, A., Sakai, F., & Kadooka, Y. Lactobacillus gasseri SBT2055 inhibits adipose tissue inflammation and intestinal permeability in mice fed a high-fat diet. *J. Nutri. Sci.* **5**, e23 (2016).
23. Parlee, S. D., Lentz, S. I., Mori, H. & MacDougald, O. A. Quantifying size and number of adipocytes in adipose tissue. *Methods. Enzymol.* **537**, 93-122 (2014).
24. Sato M. et al. Effects of milk fermented by Lactobacillus gasseri SBT2055 on adipocyte size in rats. *Br. J. Nutr.* **99**, 1013–1017 (2008).
25. Reginensi S.M., Olivera J.A., Bermúdez J. & González M.J. *Lactobacillus* in the Dairy Industry: From Natural Diversity to Biopreservation Resources. *Microorganisms for Sustainability* **1**, 57-81(2016).
26. Boumis, E., Capone, A., Galati, V., Venditti, C. & Petrosillo, N. Probiotics and infective endocarditis in patients with hereditary hemorrhagic telangiectasia: a clinical case and a review of the literature. *BMC. Infect. Dis.* **18**, 65 (2018).
27. Kadooka, Y. et al. Effect of Lactobacillus gasseri SBT2055 in fermented milk on abdominal adiposity in adults in a randomised controlled trial. *Br. J. Nutr.* **110**, 1696-1703 (2013).
28. Kadooka, Y. et al. Regulation of abdominal adiposity by probiotics (Lactobacillus gasseri SBT2055) in adults with obese tendencies in a randomized controlled trial. *Eur. J. Clin. Nutr*. **64**, 636–643 (2010).
29. Wu, G. D. et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**,105-108 (2011).
30. David, L. A. et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559-563 (2014).
31. Astrup, A. Macronutrient balances and obesity: the role of diet and physical activity. *Public. Health. Nutr.* **2**, 341-347 (1999).
32. Almeida-Suhett, C. P., Scott, J. M., Graham, A., Chen, Y. & Deuster, P. A. Control diet in a high-fat diet study in mice: Regular chow and purified low-fat diet have similar effects on phenotypic, metabolic, and behavioral outcomes. *Nutr. Neurosci.* **22**, 19-28 (2019).
33. Klop, B., Elte, J. & Cabezas, M. Dyslipidemia in obesity: mechanisms and potential targets. *Nutrients* **5**, 1218-1240 (2013).

The role of activins in TGFβ profibrotic signaling in chronic kidney disease

Snow Wangding[1]
[1]Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Canada

Abstract
Chronic kidney disease (CKD) is a global health condition affecting upwards of 14% of individuals worldwide. Characterized by stages of decreased kidney function and increased albuminuria, dialysis and kidney transplantation are the only existing therapies. Current research has established the role of TGFβ profibrotic signaling in a dysregulated wound healing response, resulting in the excessive accumulation of extracellular matrix components in kidney glomeruli and interstitium. This accumulation detrimentally and often irreversibly affects kidney function. While efforts have been made to investigate the efficacy and feasibility of blocking TGFβ1 as a therapeutic target, the consequences of inhibition result in many adverse side effects. Recent studies have identified a potential role for activins in profibrotic signaling. As cytokines in the TGFβ superfamily, activins play an important role in cell growth and differentiation and are shown to fine-tune fibrotic pathways. Here, we review the mechanisms of action involving activins and renal disease.

Introduction

Chronic kidney disease (CKD) is a rising health problem characterized by kidney damage and reduced glomerular filtration rate persisting for over 3 months[1]. The global increase in the incidence and prevalence of CKD is driven by corresponding surges in diabetes mellitus, hypertension, obesity, and aging. CKD is often asymptomatic in its early stages but can be clinically identified by abnormalities in kidney structure and increasing albuminuria as the disease progresses[1]. Pathogenically, the excessive accumulation of extracellular matrix (ECM) components in the glomeruli and tubular interstitium results in renal fibrosis and interstitial scarring which alter and affect renal structures[2]. This accumulation is primarily accomplished by the conversion of quiescent fibroblasts to active myofibroblasts which deposit ECM[3]. The resultant nephron loss and scarring ultimately lead to compromised function and may eventually lead to end-stage renal failure, for which there is no current cure. Dialysis and kidney transplantation are the only treatments for end-stage kidney disease, yet these involve complicated measures with varying levels of success. The increasing disparities between organ supply and demand further highlight the need for a method of reversing renal fibrosis[4].

Transforming growth factor beta (TGFβ) has been identified as an important mediator of the renal fibrotic process, particularly in the conversion of quiescent to active fibroblasts. Despite its role as a major profibrotic cytokine, TGFβ cannot be targeted clinically in light of its well-documented pleiotropic nature, specifically the potential abrogation of its anti-inflammatory and anti-tumorigenesis properties which would result in adverse effects. In fact, in studies using TGFβ1-deficient mice, massive inflammation resulted in mortality[5,6]. Therefore, investigation into alternative TGFβ superfamily ligands is crucial in determining potential mediators of renal fibrosis and more feasible therapeutics. More recently, activins have been found to contribute to TGFβ profibrotic effects; however, the mechanism and magnitude by which they do so remains unclear[7]. Thus, this review aims to better understand how activins contribute to renal fibrosis and elucidate their potential as a therapeutic target for CKD.

Activins as Profibrotic Cytokines

Activins belong to the TGFβ superfamily of cytokines and are important regulators of cell growth and differentiation[7]. Their structure consists of homo- or heterodimerized inhibin β subunits linked by disulphide bonds[7]. Four β subunits have been identified in mammals: βA, βB, βC, and βE. Transcripts for βA and βB are expressed in nearly all tissue, whereas the latter two are predominantly found in the liver. Activin A and Activin B, homodimers composed of inhibin βA and βB subunits respectively, have been studied more widely. Activin A associates with activated type I receptor ALK4 while Activin B binds to ALK3[7,8]. Interestingly, Activin A is not detected in normal adult kidneys, but is upregulated in renal tubular cells following ischemia and reperfusion injury[9]. Studies have shown that Activin A promotes cell proliferation and enhances type I collagen mRNA expression in primary cultured renal interstitial fibroblasts, essentially identifying Activin A as a profibrotic factor[9,10]. Together, these findings provide insight into the role of Activin A in renal fibrosis and suggest therapeutic potential for CKD treatment.

Activins transduce their signals through complexes consisting of heteromeric type I and II receptors and downstream effectors. The N-terminal domains of type I and II receptors engage in ligand binding while their C-terminal domains exhibit serine/threonine kinase activity. Activins initially bind to type II receptors (ACVR2A/B or ActRIIA/B) which phosphorylate the GS domains of recruited type I receptors, thereby activating them[7]. Examining the interactions of activin receptors has introduced a novel approach to understanding the mechanism behind profibrotic activin action.

Mechanism of Activins in TGFβ Signaling

The contribution of activins to TGFβ fibrotic signaling has yet to be clearly elucidated; however, some studies have shown a link between the regulation of activins and TGFβ pathway components. An early study used an activin receptor-inducible cell line, KAR6, and induced receptor expression to determine how activin receptors were interacting with TGFβ signal transduction. Through transfections of TGFβ pathway specific signal transducers Smad2/3, Smad4, and Smad7, it was found that ALK4 associated with Smad2/3, but not Smad4[11]. These results established that Activin A receptors mirror the role of TGFβ receptors in the canonical fibrotic response by activating the Smad signal transduction pathway (Figure 1). Upon phosphorylation, Smad2/3 multimerizes with Smad4 and the complex translocates to the nucleus to affect transcription of target genes[11].

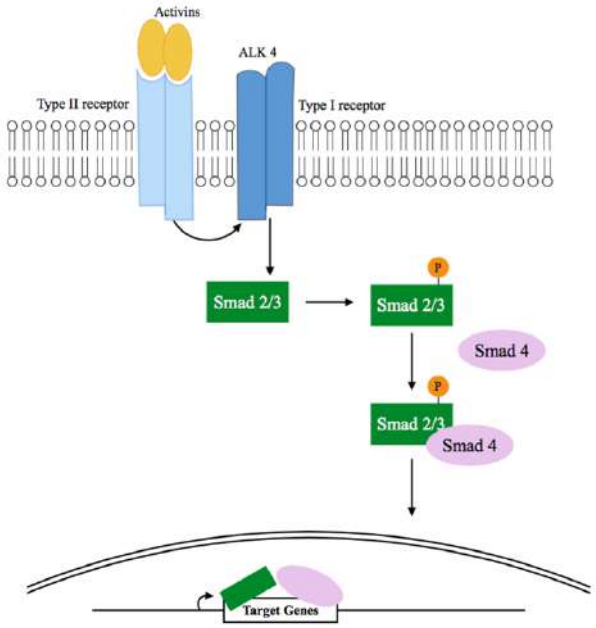


Figure 1: Canonical activin signal transduction in TGFβ profibrotic response. Activin receptors are stimulated by activins (orange). Type II receptor phosphorylates type I (ALK4 in the case of Activin A), and initiates Smad-dependent transcription of target genes. Upon binding of Smad 4 to the phosphorylated Smad 2/3 complex, nuclear localization occurs. Smads bind to target genes to facilitate transcription. Adapted from Tsuchida *et al.* (2009)[7].

Recent studies have enhanced our understanding of the degree of activin contribution to TGFβ renal fibrosis, with a particular emphasis on Activin A. Inhibiting activin receptor function has been demonstrated to decrease downstream effects of TGFβ signaling. In a study examining the effects of ActRIIA inhibition on Smad activation, treatment with the ligand trap RAP-011 decreased phosphorylation of the Smad2/3 complex[12]. This suggests that inhibition of type II activin receptors may play a role in diminishing the activation of profibrotic Smad-responsive genes. This decreased fibrotic response is supported in CKD-induced mouse models, in which renal interstitial fibrosis was significantly decreased in kidney sections of ActRIIA-inhibited groups. Inhibition of ActRIIA decreased proteinuria, a symptom of CKD, demonstrating a clinical application of activin receptor targeting[12]. Other studies have further supported these findings. For instance, in a study using truncated type II activin receptors (tARII), translocation of Smad 2/3 into the nucleus was not observed in tARII transfected cells[10]. Furthermore, inhibition of ALK4 association with Smad2/3 has been shown to decrease transcriptional activity of activin-induced target genes[11]. Taken together, these findings implicate the contribution of activins to profibrotic Smad-induced transcription. However, the magnitude of activin contribution relative to TGFβ receptor signaling remains unclear, specifically because both cytokines use Smad-activated signal transduction. In kidney mesangial cells, our group found that Activin A and B inhibition significantly inhibits the profibrotic response induced by TGFβ1 treatment[13]. Using the natural antagonist of activins, follistatin does not inhibit TGFβ1, thus these results aid in clarifying the relative role of activins to TGFβ-induced fibrosis. Further confirming this finding, we used an anti-Activin A antibody for more targeted inhibition and found decreased Smad3 phosphorylation and transcriptional activity. This suggests that activin inhibition may be attenuating the response initiated by TGFβ receptor signaling.

Few studies have focused on the role of Activin B in TGFβ profibrotic signaling. In polycystic kidney disease (PKD), the inhibition of Activin receptor type-2B (ActRIIB) shows similar effects in inhibiting cyst progression. PKD is a type of CKD in which cyst growth prevents kidney function and can lead to

renal failure[14]. PKD phenotype is associated with increased proliferation and expression of Smad2/3 target genes, relating its development to the activation of TGFβ fibrosis[14]. In a study examining ActRIIB-inhibited mice, the inhibited groups showed reduced expression of type I and α1 collagen, as well as decreased total Smad2 levels[15]. In another study examining the inhibition of ActRIIB in acute kidney injury, it was found that Activin A receptor inhibition resulted in decreased accumulation of fibroblasts, which are an indicator of developing fibrosis[16]. These findings suggest that Activin A may play a role in contributing to Smad2/3-dependent signaling and fibrosis.

Between Activin A and Activin B, Activin A is the more elucidated cytokine in reference to the contribution of activins to TGFB-induced renal fibrosis. Our group found that there was a notable 18-fold increase in Activin A secretion in cells treated with TGFβ1. Comparatively, there was only a 0.5-fold increase in Activin B secretion. Additionally, we found that inhibition of Activin B using an anti-Activin B antibody had no effect on the transcriptional activity of an established marker of myofibroblast differentiation, αSMA. In current literature, these findings suggest that while Activin B may play a role in the TGFβ profibrotic response, Activin A is much more relevant in investigating the potential downstream effects of activins in fibrotic signaling. Interestingly, our group found that when Activin A was added to TGFβ1-treated cells, there was no significant increase in the transcriptional activity of Smad3[13]. This suggests that activins may also be contributing to renal fibrosis in a non-canonical (non-Smad3 dependent) manner. In the complexity and layered mechanisms of TGFβ profibrotic signaling, developing a more comprehensive understanding of activin-induced downstream effects and transcriptional interactions is crucial. This will be further explored below.

Transcription Factors and αSMA

Mentioned previously, TGFβ is critical in the activation of myofibroblasts, cells which deposit ECM components in pathogenic renal fibrosis. In kidney glomeruli, activated mesangial cells exhibit myofibroblast cell characteristics pertaining to their ECM production and contractility[17]. Thus, activated mesangial cells are important models for understanding the conversion of inactive fibroblasts to active myofibroblasts. A distinct and well-documented marker of glomerular disease in activated mesangial cells is alpha smooth muscle actin (αSMA). While normally expressed in adult smooth muscle and transiently in cardiac and skeletal muscle, expression of αSMA is also a prominent feature of differentiated myofibroblasts[18,19]. In mice, pulmonary and renal fibrosis were significantly associated with the accumulation of αSMA-producing myofibroblasts[20]. Together, this makes αSMA an appropriate marker for renal fibrosis.

In the canonical profibrotic response, stimulated TGFβ receptors phosphorylate Smad2/3 and enable the multimerization with Smad4. Smad4 is crucial for signal transduction, as loss of Smad4 in mesangial cells inhibits TGFβ-induced ECM component accumulation and deposition[21]. The activated complex consisting of Smad2/3 and cofactor Smad4 then translocates to the nucleus and acts as a transcriptional factor in binding to Smad-specific DNA binding motifs[22]. Notably, Smad3 directly binds to regions within the αSMA promoter, called Smad-binding elements (SBE)[19]. In addition to Smad3, other binding factors impact the transcriptional activity of αSMA. In fact, the yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are transcriptional cofactors that have been shown to contribute to fibrosis. YAP has been shown to associate with Smad7, an inhibitory Smad in the TGFβ signaling pathway, and also Smad3 in mesothelioma cells [23,24]. In addition, YAP has been shown to be induced by TGFβ treatment, implying a role in fibrosis[25].

YAP/TAZ in Fibrosis

YAP and TAZ are important regulators of organ size, stem cell fate, and solid tumour growth, yet little is known regarding their mechanism of action[23]. Studied primarily in the context of the Hippo signaling cascade, YAP/TAZ have been shown to localize to the nucleus when dephosphorylated. YAP and TAZ transcription factors may bind to different DNA-binding proteins, but both associate with TEA DNA-binding domain (TEAD) elements in the αSMA promoter region[18,22]. This implicates their role in renal fibrosis. In addition to phosphorylation, the regulation of YAP/TAZ activity is mechanosensitive. Recently studied, ECM stiffness has been found to modulate the localization of YAP/TAZ based on cytoskeletal tension. In healthy tissue, cytoskeletal tension is reduced and YAP/TAZ localize to the cytoplasm. However, YAP/TAZ have been found to localize to the nucleus in cells grown on stiff and injured surfaces[3]. This is seen in studies examining idiopathic pulmonary fibrosis, mammary epithelial cells, HeLA cells, and kidney fibroblasts[3,23-24]. Stiff tissue is characteristic of fibrosis, as the accumulation of ECM components causes the ECM to stiffen. In a particular study using stiff substrates

to model injured fibrotic kidneys, immunostaining revealed the localization of YAP/TAZ to the nucleus[3]. This suggests that the presentation of fibrosis involves the activation and thus nuclear localization of YAP and TAZ. Other studies have clearly demonstrated this effect[30]. In pulmonary fibrosis, YAP and TAZ levels have been shown to be elevated[30–32]. Knockdown studies have also found reduced pro-collagen, αSMA, and lowered levels of proteins responsible for myofibroblast differentiation[23-24]. Together, these findings strongly imply that YAP and TAZ induce renal fibrosis through activation of myofibroblasts in a mechanosensitive manner.

YAP/TAZ Interaction with Smads

Recent studies with YAP/TAZ inhibitors have demonstrated a relationship between ECM stiffness and TGFβ-induced SMAD localization mediated by YAP/TAZ[3,28]. TGFβ treatment induced equivalent Smad2/3 phosphorylation in fibronectin substrates modelling healthy and damaged tissue. Soft fibronectin substrates were used to model healthy tissue, and stiff substrates for damaged tissue. However, localization of Smad2/3 to the nucleus was diminished in cells grown on soft substrates, where Smad-induced transcriptional activity was also reduced. These findings indicate that ECM stiffness mediates Smad2/3 localization without affecting TGFβ-induced phosphorylation[3]. This effectively implies that a positive feedback loop drives pathological fibrosis[28]. YAP/TAZ are implicated in driving this mechanism. Smad2/3 TGFβ-stimulated accumulation in the nucleus was shown to be decreased upon inhibition of YAP/TAZ using verteprofin treatment. Verteprofin had no effect on TGFβ-induced phosphorylation of Smads. In addition, the inhibition of YAP/TAZ resulted in attenuation of Smad3 transcriptional activity and endogenous expression of Smad-inducible collagen genes[3]. These findings suggest possible crosstalk between YAP/TAZ and Smad-dependent pathways modulating TGFβ fibrosis.

Our group found that activin inhibition with follistatin prevented TGFβ1-induced YAP activation (Figure 2)[13]. As follistatin does not inhibit TGFβ, this response suggests that activins indeed play a substantial role in TGFβ profibrotic signalling by perhaps regulating YAP/TAZ activation. A study examining TAZ expression in human kidney cells showed that TAZ silencing neutralized TGFβ1-induced expression of target genes without affecting Smad3 phosphorylation[26]. This suggests that TAZ is a non-Smad downstream effector of renal fibrosis, implying that non-canonical pathways may be significantly involved in the regulation of TGFβ profibrotic events. Another study also supports the Smad3-independent transcriptional activity of TAZ[33]. In this study, fibroblast cells were treated with a Smad3 inhibitor, SIS3, before being treated with TGFβ. In addition to the Smad3 inhibitor, a separate experiment was conducted using Smad3 siRNA prior to TGFβ treatment. Both groups failed to reduce TAZ expression, suggesting that TAZ is mediated by non-canonical pathways. In contrast, a study using pig kidney cells demonstrated that TAZ confers Smad3 sensitivity to the αSMA promoter by associating with SBE[34]. Where Smad3 alone was not sufficient to induce activation of the αSMA promoter, the presence of TAZ allowed for a synergistic effect[34]. Taken together, these findings further support the implication that activins could be mediating YAP/TAZ activation in both canonical and non-canonical mechanisms of action.

Conclusion

CKD is a health problem characterized by renal fibrosis and prolonged kidney dysfunction affecting filtration and waste clearance. CKD progresses rapidly and irreversibly, eventually causing death if left untreated. The predominant cytokine upregulating fibrosis, TGFβ, transduces its signal through a Smad-dependent signalling cascade; however, TGFβ cannot be targeted clinically due to adverse side effects. Major advancements have been made in understanding the role of activins in TGFβ-induced renal fibrosis[8,9,13,15]. With recent studies suggesting that activins may contribute significantly to the canonical and non-canonical processes of fibrosis, more investigation is required into the complexities of how activins interact with YAP/TAZ transcription factors to develop feasible targets to reverse CKD renal fibrosis.

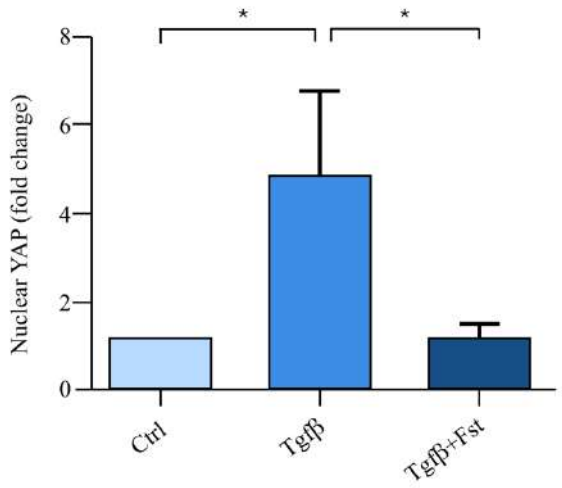


Figure 2: Activin inhibition with follistatin (Fst) prevents TGFβ-induced nuclear YAP activation.

Examined from western blot data; mouse mesangial cells were treated with TGFβ-1 and TGFβ-1 with Fst over 18h. Significantly reduced nuclear YAP expression with Fst observed, n=9, t-test *p < 0.05. Adapted from Soomro *et al.* (2019)[13].

Acknowledgements

Special acknowledgements to Dr. Krepinsky for the opportunity to pursue her undergraduate thesis investigating renal disease at St. Joseph's Hospital, Hamilton. I also want to thank my lab mentor, PhD candidate Asfia Soomro. This work was completed as per the requirements of BIOCHEM 4F09, Fall 2019, at McMaster University.

References

1. Jha, V. *et al.* Chronic kidney disease: Global dimension and perspectives. *The Lancet* **382**, 260–272 (2013).
2. López-Hernández, F. J. & López-Novoa, J. M. Role of TGF-β in chronic kidney disease: An integration of tubular, glomerular and vascular effects. *Cell and Tissue Research* **347**, 141–154 (2012).
3. Szeto, S. G. *et al.* YAP/TAZ Are Mechanoregulators of TGF-b-Smad Signaling and Renal Fibrogenesis. *J. Am. Soc. Nephrol.* **27**, 3117–3128 (2016).
4. Fraser, S. & Blakeman, T. Chronic kidney disease: identification and management in primary care. *Pragmatic Obs. Res.* **Volume 7**, 21–32 (2016).
5. Walton, K. L., Johnson, K. E. & Harrison, C. A. Targeting TGF-β mediated SMAD signaling for the prevention of fibrosis. *Frontiers in Pharmacology* **8**, (2017).
6. Meng, X. M., Tang, P. M. K., Li, J. & Lan, H. Y. TGF- β/Smad signaling in renal fibrosis. *Frontiers in Physiology* **6**, (2015).
7. Tsuchida, K. *et al.* Activin signaling as an emerging target for therapeutic interventions. *Cell Commun. Signal.* **7**, (2009).
8. Protic, O. *et al.* Activin A in Inflammation, Tissue Repair, and Fibrosis: Possible Role as Inflammatory and Fibrotic Mediator of Uterine Fibroid Development and Growth. *Semin. Reprod. Med.* **35**, 499–509 (2017).
9. Fang, D. Y. P. *et al.* The Role of Activin A and B and the Benefit of Follistatin Treatment in Renal Ischemia-Reperfusion Injury in Mice. *Transplant. Direct* **2**, e87 (2016).
10. Yamashita, S., Maeshima, A., Kojima, I. & Nojima, Y. Activin A Is a Potent Activator of Renal Interstitial Fibroblasts. *J. Am. Soc. Nephrol.* **15**, 91–101 (2004).
11. Lebrun, J. J., Takabe, K., Chen, Y. & Vale, W. Roles of pathway-specific and inhibitory Smads in activin receptor signaling. *Mol. Endocrinol.* **13**, 15–23 (1999).
12. Agapova, O. A., Fang, Y., Sugatani, T., Seifert, M. E. & Hruska, K. A. Ligand trap for the activin type IIA receptor protects against vascular disease and renal fibrosis in mice with chronic kidney disease. *Kidney Int.* **89**, 1231–1243 (2016).
13. Soomro, A., Mehta, N., Gao, B. & Krepinsky, J. A New Therapeutic Target for CKD: Activins Facilitate TGF-β1 Profibrotic Signaling in Kidney Mesangial Cells. (2019). Available at: https://www.asn-online.org/education/kidneyweek/2019/program-abstract.aspx?controlId=3226890. (Accessed: 25th October 2019)
14. Bleyer, A. J., Hart, T. C. & Wilson, P. D. Polycystic kidney disease. *New Engl. J. Med.* **350**, 2622 (2004).
15. Leonhard, W. N. *et al.* Inhibition of Activin Signaling Slows Progression of Polycystic Kidney Disease. *J. Am. Soc. Nephrol.* **27**, 3589–3599 (2016).
16. Palin, N. K. *et al.* Activin inhibition limits early innate immune response in rat kidney allografts—a pilot study. *Transpl. Int.* **30**, 96–107 (2017).
17. Kawada, N. *et al.* Role of intron 1 in smooth muscle α-actin transcriptional regulation in activated mesangial cells in vivo. *Kidney Int.* **55**, 2338–2348 (1999).
18. Boukhalfa, G., Desmoulière, A., Rondeau, E., Gabbiani, G. & Sraer, J. D. Relationship between alpha-smooth muscle actin expression and fibrotic changes in human kidney. *Exp. Nephrol.* **4**, 241–247 (1996).
19. Hu, B., Wu, Z. & Phan, S. H. Smad3 Mediates Transforming Growth Factor-Induced-Smooth Muscle Actin Expression. *Am. J. Resp. Cell. Mol.* **29**, (2003).
20. Lebleu, V. S. *et al.* Origin and function of myofibroblasts in kidney fibrosis. *Nat. Med.* **19**, 1047–1053 (2013).
21. Tsuchida, K. I., Zhu, Y., Siva, S., Dunn, S. R. & Sharma, K. Role of Smad4 on TGF-β-induced extracellular matrix stimulation in mesangial cells. *Kidney Int.* **63**, 2000–2009 (2003).
22. Schiller, M., Javelaud, D. & Mauviel, A. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J. Derma-tological Sci.* **35**, (2004).
23. Piersma, B., Bank, R. A. & Boersema, M. Signaling in fibrosis: TGF-β, WNT, and YAP/TAZ converge. *Frontiers in Medicine* **2**,

(2015).
24. Fujii, M. *et al.* Convergent signaling in the regulation of connective tissue growth factor in malignant mesothelioma: TGFβ signaling and defects in the Hippo signaling cascade. *Cell Cycle.* **11**, 33373-9 (2012).
25. Piccolo, S., Dupont, S. & Cordenonsi, M. The Biology of YAP/TAZ: Hippo Signaling and Beyond. *Physiol. Rev.* **94**, 1287–1312 (2014).
26. Anorga, S. *et al.* Deregulation of Hippo-TAZ pathway during renal injury confers a fibrotic maladaptive phenotype. *FASEB J.* **5**, 2644-2657 (2018).
27. Zhao, B. *et al.* TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev*. **22**, 1962-1967 (2008).
28. Liu, F. *et al.* Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **308**, L344–L357 (2015).
29. Mannaerts, I. et al. The Hippo pathway effector YAP controls mouse hepatic stellate cell activation. *J. Hepatol.* **3**, 679-688 (2015).
30. Noguchi, S., Saito, A. & Nagase, T. YAP/TAZ signaling as a molecular link between fibrosis and cancer. *Int. J. Mol.* **19**, (2018).
31. Seo, E. *et al.* The Hippo-Salvador signaling pathway regulates renal tubulointerstitial fibrosis. *Sci. Rep.* **6**, (2016).
32. Xu, J. *et al.* Involvement of the hippo pathway in regeneration and fibrogenesis after ischaemic acute kidney injury: YAP is the key effector. *Clin. Sci.* **130**, 349–363 (2016).
33. Miranda, M. Z. *et al.* TGF-β1 regulates the expression and transcriptional activity of TAZ protein via a Smad3-independent, myocardin-related transcription factor-mediated mechanism. *J. Biol. Chem.* **292**, 14902–14920 (2017).
34. Speight, P., Kofler, M., Szászi, K. & Kapus, A. Context-dependent switch in chemo/mechanotransduction via multilevel crosstalk among cytoskeleton-regulated MRTF and TAZ and TGFβ-regulated Smad3. *Nat. Commun.* **7**, (2016).

Exploring Misconceptions Surrounding Vaccinations: A Health Advocacy Project

Freeman Paczkowski[1], Parisa Agahi[1], Anson Lee[1], Divya Tamilselvan[1]
[1]Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Canada

Abstract
Despite a consistent effort by the scientific community to inform individuals about the efficacy and safety of vaccinations, harmful misconceptions still persist which have prevented their widespread acceptance within the general public. According to the most recent survey from the Public Health Agency of Canada (2017), childhood vaccination rates (children aged two years or less) were approximately 70-90% depending on the type of vaccine, and in each province, were below the national goal[1]. Additionally, the Canadian childhood vaccination rates reported for the common vaccines diphtheria, tetanus, pertussis, and measles were reported to be some of the lowest in the developed world[2]. To better understand these disparities, this video explores common misconceptions individuals may have toward vaccines. Specifically, this project takes the form of a skit which explores the perspectives of a female high school student, a mother of young children, and a male university student to understand their skepticism towards the HPV, MMR, and flu vaccines respectively. Overall, the aim of this project is to better inform the public about the benefits of vaccines in order to increase vaccination rates in Canada and improve health care outcomes for infectious diseases that are preventable by vaccines.

For more information regarding the safety, efficiency, and accessibility of vaccines, please contact your local health-care provider, as well as refer to these online resources: Immunize Canada (https://immunize.ca/resources); Infection Prevention and Control Canada (https://ipac-canada.org/immunization-resources-2.php); and The Canadian Vaccine Catalogue (https://cvc.canimmunize.ca/en/home).

An Interview with Vaccine Skeptics Video: https://youtu.be/CtWcj_13dZ8

Acknowledgements
This project was initially completed as coursework for BIOMEDDC 3A03-Road to Biomedical Discovery in the fall of 2019. We would like to formally thank the professor of BIOMEDDC 3A03 Dr. Matthew Miller, the teaching assistant Hannah Stacey, as well as the BDC program administration for organizing an incredible course and providing us with a truly enriching academic experience. Additionally, we acknowledge that the graphics used for this video were created using Apple iMovie.

Abstract References

1. Vaccine Coverage in Canadian Children: Results from the 2015 Childhood National Immunization Coverage. https://www.canada.ca/en/public-health/services/publications/healthy-living/2017-vaccine-uptake-canadian-children-survey.html (2018).
2. Child vaccination rates. https://data.oecd.org/healthcare/child-vaccination-rates.htm (2018).

Video References

1. Bird, Y., et. al. Human Papillomavirus Vaccination Uptake in Canada: A Systematic Review and Meta-analysis. *Int J Prev Med.* **8**, (2017).
2. Mayo Clinic: HPV Infection. https://www.mayoclinic.org/diseases-conditions/hpv-infection/symptoms-causes/syc-20351596 (2018).
3. Immunize Action Collection: Autism. http://www.immunize.org/autism/ (2018).
4. Ontario Ministry of Health and Long-Term Care: Publically Funded Immunization Schedules for Ontario in 2016. http://www.health.gov.on.ca/en/pro/programs/immunization/docs/immunization_schedule.pdf (2018).
3. Health Link BC: Influenza (Flu) Immunization: Myths and Facts. https://www.healthlinkbc.ca/healthlinkbc-files/flu-vaccine-myth-facts (2015).

Malaria: An Overview of Past and Current Strategies to Attain Attenuation and Eradication

Pegi Kaimi[1]
[1]Department of Biochemistry and Biomedical Sciences, Faculty of Health Sciences, McMaster University, Canada

Abstract
As antimicrobial resistance continues to rise, infectious diseases that were once thought to be easily treated are now re-emerging with greater strength. Malaria, a deadly vector-borne disease, continues to be prevalent in predominantly developing countries, despite the numerous attempts that have been undertaken to develop new strategies for elimination and eradication. This literature review will highlight the past and current approaches that have been developed for malaria eradication purposes, and future strategies that emphasize the importance of the mosquito gut microbiota in discovering novel small molecules to attenuate transmission.

Introduction

The staggering proliferation of antibiotic resistance over the past few decades has led to the re-emergence of devastating infectious diseases, such as malaria. It is estimated that approximately half of the world's population is at risk of contracting malaria, with 219 million cases and 435,000 deaths reported in 2017[1]. While cases have been documented from 87 countries, 92% of these instances originate from the WHO African region. In Africa, 99.7% of cases are caused by the deadliest and most common form of the six plasmodial species that infect humans, known as *Plasmodium falciparum*[1–3]. Although this parasite begins as a mild febrile disease, it often becomes severe in individuals with an underdeveloped or compromised immune system, as in young children and pregnant women[4,5]. These individuals may develop cerebral malaria and severe anemia, which can potentially cause death[2,3,6]. Therefore, there is an urgent need for new therapeutic strategies to combat resistance towards current drugs to reduce the transmission of malaria in highly affected areas[2]. This review will introduce the pathway of malaria transmission and pathogenesis by outlining the life cycle of *P. falciparum* and illustrate the potential intervention sites. Once the molecular mechanisms of the disease have been discussed, past and current approaches for attenuating the transmission of malaria will be explored, along with future directions. This literature review will additionally highlight the potential of manipulating the mosquito gut microbiota as a means to reduce vector competence, and therefore attenuate malarial transmission in this manner[7,8].

Malaria Transmission and Pathogenesis

Malaria is a vector-borne disease with three main stages: the pre-erythrocytic liver stage and asexual blood stage, which occur in the vertebral host. The third stage, known as the sexual cycle, occurs in *Anopheles* mosquitoes[9]. During a blood meal, female *Anopheles* release sporozoites from their salivary glands into the dermis of the victim[3,10,11]. The motile sporozoites will penetrate nearby blood vessels and enter the bloodstream, quickly accessing the liver via the process of traversal. This involves sporozoites crossing the sinusoidal barrier, which is composed of fenestrated endothelial cells and Kupfer cells. Transient vacuoles are then formed in association with pore-forming proteins: SPECT (sporozoites microneme protein essential for traversal), PLP1 (perforin-like protein 1) and CelTOs (cell-traversal protein for ookinete's and sporozoites)[3,11]. This leads to the penetration of hepatocytes and the initiation of the liver stage[2,3]. During this stage, sporozoites proliferate into merozoites, replicating asexually for seven days. Shortly thereafter, 40,000 merozoites per hepatocyte are released into the bloodstream, resulting in the invasion of erythrocytes and establishment of the asexual blood stage (Figure 1)[2,12].

The intracellular proliferation of the parasite during the asexual blood stage confers parasite protection against host immune responses. It is also responsible for causing indicative malarial symptoms, such as fever and tremors[2,11]. During this stage, the parasite creates significant changes to erythrocytes. Infected erythrocytes develop digestive vacuoles that house vital amino acids and assist in the digestion of hemoglobin to support parasite growth and development[9]. Subsequently, infected erythrocytes are remodeled through the surface presentation of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). The presentation of this surface protein allows infected erythrocytes to bind to endothelial cells in order to avoid spleen clearance[2,6]. During the blood stage, merozoites develop through three stages and asexually replicate within the infected erythrocytes. Schizonts are ultimately formed, rupturing erythrocytes and

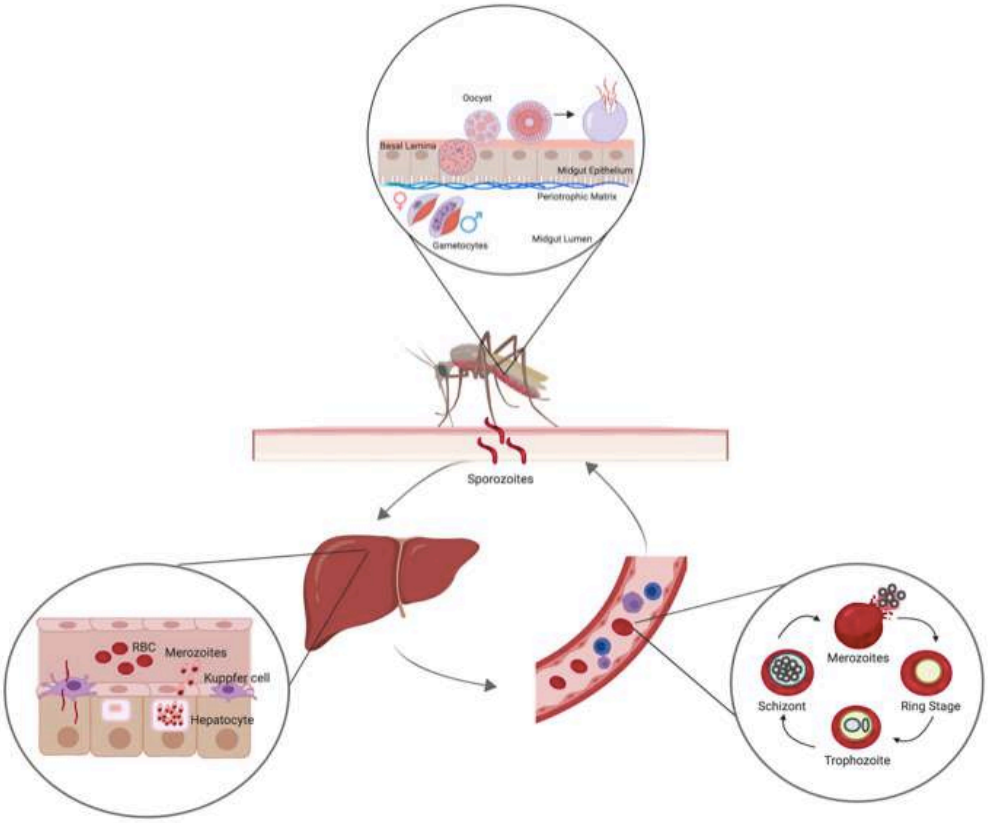


Figure 1. An illustration of the P. falciparum lifecycle within the vertebral host and the mosquito vector. Sporozoites are released into the bloodstream and undergo traversal to establish the liver stage. Sporozoites develop into merozoites within liver cells and are subsequently released into the bloodstream, establishing the asexual blood stage. Merozoites undergo three stages of development: ring stage, trophozoite, and eventually become schizonts. This cycle continuously propagates, with a small proportion of asexual schizonts becoming gametocytes. During subsequent blood meals, uninfected mosquitoes ingest gametocytes, which undergo gametogenesis and fertilization in the mosquito gut. This results in a zygote, which is made into an ookinete that can penetrate the midgut epithelium and become an oocyte. 5 oocytes are made, which subsequently make 50,000 sporozoites that infect the salivary glands of the mosquito, therefore ensuring the propagation of the parasite. Adapted from Cowman *et al.* (2016), Dong *et al.* (2009), and Aoki *et al.* (2017)[3,51,65].

resulting in anemia and the spread of the parasitic infection[3]. Throughout this proliferative process, a small proportion of parasites differentiate into male and female gametocytes. These gametocytes undergo five stages of development in the extravascular space of the bone marrow[12]. The victim becomes infectious once male and female gametocytes (Stage V) re-enter the peripheral circulation[12].

The propagation of *P. falciparum* relies on the transmission of gametocytes from an infected vertebral host to non-infected mosquitoes through blood-feeding[12]. As gametocytes from the blood meal enter the mosquito gut lumen, environmental factors, such as the presence of xanthuric acid, trigger gametogenesis. The result is the formation of male microgametes and female macrogametes, which undergo fertilization to form zygotes[10,12]. Within 24 hours, the zygotes differentiate into motile ookinetes that invade the mosquito midgut epithelium. After crossing the epithelium, ookinete's develop into oocysts which reside below the basal lamina of the midgut wall[10,13,14]. At this stage, the parasite experiences a severe bottleneck, where only five ookinetes are able to traverse the gut epithelium and develop into oocysts, making the midgut an important site for intervention[15,16]. It is important to note that the epithelial gut lining (peritrophic matrix), which is composed of chitin fibrils and glycoproteins, has been implicated in the bottleneck experienced in the midgut[17,18]. Current research suggests that the peritrophic matrix could be targeted to attenuate ookinete traversal fully[16]. Alteration of the mosquito's midgut microbiota may assist in this initiative, as mosquitoes can be fed factors that induce biofilm formation. The creation of a biofilm would make it more difficult for the ookinete to traverse the midgut epithelium.

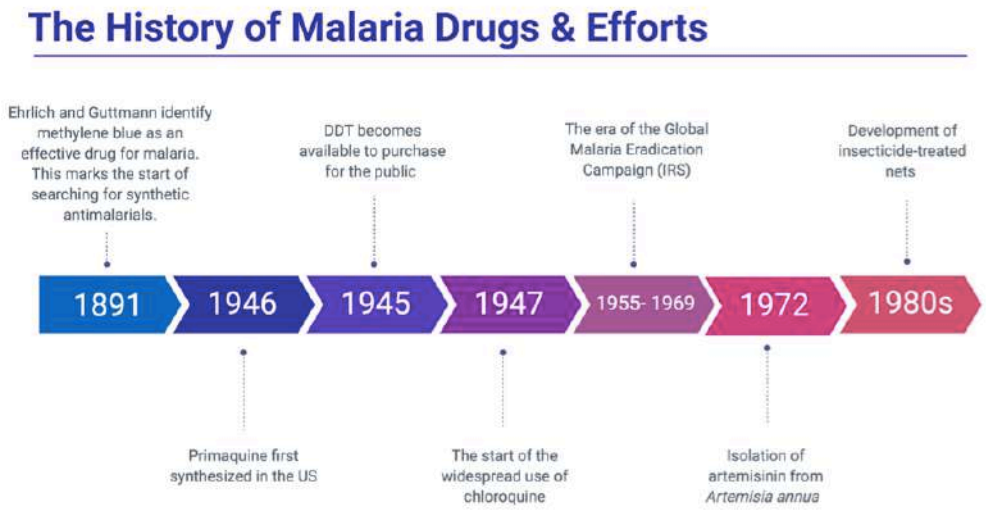


Figure 2. A description of the compound-based drugs developed throughout history and the efforts taken to eradicate malaria. An important discovery involved the identification of methylene blue as an effective drug to treat malaria. This discovery spurred the development of synthetic antimalarials, ultimately leading to the identification of chloroquine and artemisinin. Adapted from Wells et al. (2015), Center for Disease Control and Prevention (2019), Prevention, C (2019), and Aoki *et al.* (2017)[62-65].

Nevertheless, successful oocysts undergo a maturation process to produce sporozoites, which are subsequently released into the mosquito hemocoel (body cavity). Ten to fourteen days after the initial blood meal, mosquitoes become infectious due to sporozoite invasion of the salivary glands, allowing for the efficient transfer of sporozoites into vertebrate hosts in subsequent blood meals[12,14]. Due to the complex life cycle of *P. falciparum*, many intervention sites exist and can be coopted to attenuate malaria transmission.

Past and Current Approaches in Treating and Preventing Malaria

Since the twentieth century, many approaches have been developed to attenuate malaria, either targeting the parasitic forms within the vertebrate host or coopting the mosquito vector to prevent the completion of the sexual cycle. Additionally, the development of prevention strategies, such as utilizing insecticide-treated nets and promoting indoor residual spraying (IRS), has assisted in the reduction of malaria cases in highly endemic areas[19]. IRS reduces the female mosquito lifespan, thereby limiting its transmission capacity, which ultimately decreases mosquito vector density[19,20]. However, concern surrounding the possibility of resistance towards insecticides and prevention strategies has driven research efforts towards elucidating novel ways to eradicate malaria[7].

Malarial Drugs in the Past and Present

Malaria drug discovery has sought to identify compounds that target the asexual blood-stage. These compounds are highly desired as they can improve host survival, disease symptoms, and reduce the risk of progression towards severe malaria[2]. However, drugs have also been developed to target gametocytes formed during the blood-stage. The first compound discovered to treat malaria is known as quinine, an alkaloid isolated from the bark of the chinchona tree[21]. Quinine functions by eliminating asexual schizonts during the blood stage[19]. Despite its therapeutic activity, quinine presented significant challenges involving poor tolerability and poor compliance with dosing procedures[21]. As a result, there was an increased demand to find quinine substitutes in the early twentieth century, which led to the discovery of 8-aminoquinolone primaquine and 4-aminoquinolone chloroquine[22]. The discovery of these compounds was partly due to previous work conducted by Ehrlich with methylene blue[2].

Chloroquine is thought to target asexual parasites in infected erythrocytes by interrupting hematin detoxification during hemoglobin digestion in the digestive vacuole, thereby resulting in parasite poisoning[22,23]. Although the success of chloroquine as a first-line treatment and sulphadoxine-pyrimethamine as the second-line treatment was unparalleled when used in combination with the insecticide dichloro-diphenyl-trichloroethane (DDT) in IRS, their prolific and continued use during the

mid-twentieth century eventually lead to the emergence of malarial resistance[23-25].

Chloroquine resistance is thought to have developed due to several point mutations in the *pfcrt* gene, resulting in a mutant transporter that expels chloroquine from the digestive vacuole. It is postulated that the proton motive force established by the vacuole assists in the expulsion of chloroquine, thereby rendering these strains resistant[26,27]. Despite the progress made by chloroquine and DDT in reducing mortality and morbidity, the lack of an alternative, low-cost therapeutic drug at the time resulted in a relapse of malaria cases[22].

The new push for discovering novel antimalarial compounds led to the isolation of artemisinin (ART), a sesquiterpene lactone, extracted from the sweet wormwood *Artemesia annua* in 1972 (Figure 2)[2,22,28,29]. ART function is associated with the reductive scission of its peroxide bridge by haem iron, produced in the digestive vacuole during the digestion of hemoglobin in the asexual blood stage. Additionally, other studies have associated ART with the formation of free radicals that can alter protein and lipids in the digestive vacuole. Since ART has poor tolerability and a short-half life, it is not administered as a monotherapy, but in conjunction with derivatives such as artemether or dihydroartemisinin[30]. The use of derivatives increases oral bioavailability and helps reduce selective pressure in antimicrobial resistance[24]. The combination of ART with other derivatives compounds is known as artemisinin-combination therapy (ACT), which is currently used as the first-line treatment in highly endemic areas. However, partial resistance towards ART and ACT has recently been reported[31]. In these partially resistant strains, the use of ACT results in delayed parasite clearance in the infected vertebral host; implicated with mutations in the K13 propeller domain[31]. Additionally, ACTs are only active against asexual schizonts and cannot clear late stage gametocytes, thereby retaining a vertebral host reservoir[32]. Nevertheless, due to continuous emergence of resistance in various compound-based drugs throughout history, there is an urgent need to redirect research efforts towards elucidating other methods of eradicating malaria.

The Development of a Malaria Vaccine

Due to the continuous emergence of resistance towards compound-based drugs, scientists aim to develop vaccines that confer vertebrate host protection, thereby reducing mortality. The development of a vaccine has been proposed due to evidence showcasing human acquired immunity towards malaria when such subjects were injected with irradiated sporozoites[33]. This finding has spurred further research efforts that seek to develop vaccines capable of interrupting the transmission of malaria[34]. Three stages of the parasite life cycle have been identified as potent targets for the development of plausible vaccines: pre-erythrocytic, blood stage, and transmission-blocking vaccines (TBVs).

Pre-erythrocytic vaccines seek to impede the establishment of the asexual blood stage, thereby preventing clinical malaria[35]. They prevent sporozoites from invading hepatocytes by utilizing neutralizing antibodies to produce an antibody-mediated response; likewise, they can also attempt to destroy sporozoites within infected hepatocytes through cytotoxic CD8+ T-cell responses[19,33,35]. The leading malaria vaccine, which is pre-erythrocytic and recently completed Phase III of clinical trials, is known as RTS,S/AS01E[36]. This vaccine is based on generating antibodies against the parasite circumsporozoite protein (CSP), resulting in CSP-specific CD4 T cell responses. However, no CSP-specific CD8+ T cell response has been generated[36]. Further strategies are currently being tested to improve RTS,S, such as incorporating liver- or blood-stage antigens alongside CSP or utilizing an adenovirus capable of expressing CSP and RTS,S/AS01[36]. While these efforts may lead to promising results, complete malaria eradication will require a vaccine with higher efficacy[32]. Such a vaccine can achieve greater efficacy with the addition of adjuvants, as was recently demonstrated by the addition of adjuvants Abisco®-100 and CoVaccineHT™, in a malaria vaccine tested on a mouse model of *Plasmodium berghei.* The use of these adjuvants led to a greater pool of antigen specific central memory CD8+ cells. Stimulating positive T cell responses through the use of adjuvants can increase the efficacy of a vaccine, thereby conferring individuals with full protection from a single inoculation[37].

Blood stage vaccines seek to target and inactivate merozoites within infected erythrocytes or target malaria surface antigens expressed on infected erythrocytes, with the goal of mimicking immunity[38]. These vaccines intend to minimize clinical symptoms and are generally mediated by an antibody response, although T cell responses have also been reported[35].

However, it can be difficult to develop such vaccines as *P. falciparum* evades the immune system through

antigenic variation of surface antigens such as PfEMP1[4]. Effective blood stage vaccines may need to employ various versions of the antigens to ensure the host attains some immunity towards the parasite[4].

Finally, TBVs aim to provide an antibody or complement that is ingested alongside the parasite during the mosquito blood meal, preventing the formation of a zygote in the *Anopheles* midgut[19]. Previous studies have associated TBV efficacy with the ability of ingested antibodies to bind to parasite surface antigens during the sexual cycle. Important TBV targets include Pfs25, Pfs48/45, and Pfs230[39]. Although TBVs are promising in the pursuit of malaria eradication, employing a multi-faceted approach that simultaneously targets multiple stages of the parasite life cycle may be more effective in blocking the transmission of *Plasmodium*[32].

Mosquito Vector Control – Genetic Approaches

Many past and current studies have postulated the importance of rendering mosquitoes incapable of supporting gametocyte development to attenuate the transmission of malaria[16]. One manner in which mosquitoes can be made ineffective is through transgenic modifications, whereby modified mosquitoes carry antimalarial effector genes to halt the *Plasmodium* sexual cycle. Four main antimalarial effector gene classes have been identified based on their mode of action; these classes include gene products for parasite killing, parasite interaction, gene products that interact with the mosquito midgut or salivary gland, and gene products that can manipulate the vector's immune system[16]. Gene products with a parasite killing function are typically peptides characteristic of the innate immune system of the mosquito, such as cecropins, defensins, and gambicin[40,41]. On the other hand, molecules that interact with the parasite typically prevent its transmission across the midgut epithelium[16]. Past studies have elucidated the importance of an enolase-plasminogen interaction peptide that binds to enolase on ookinete surfaces, thereby preventing its association with plasminogen; ultimately, prevention of the enolase-plasminogen interaction inhibits the ookinete's ability to traverse the midgut epithelium[42]. Previous studies have genetically engineered mosquitoes to express the salivary gland and midgut peptide 1, which is thought to interact with a putative ookinete receptor on the luminal surface of the midgut epithelium and the salivary gland membrane, inhibiting ookinete traversal[43,44].

To successfully create genetically modified mosquitoes, they must have a fitness advantage and compete for available resources with the wild vector population[45]. For successful transgenesis, gene-drive systems must be incorporated alongside antimalarial gene effectors to assist transgenic mosquitoes in infiltrating the wild vector population, thereby ensuring the inheritance of the transgene[45,46]. Gene-drive systems that have been utilized in producing genetically modified insects include the maternal-effect dominant embryonic arrest (MEDEA) and homing endonuclease gene (HEG) systems[16]. MEDEA is based on a toxin-antidote system, whereby an effector gene is linked to a miRNA toxin that is expressed during oogenesis, inactivating an essential maternal gene required for embryogenesis; a zygotic antidote, which expresses the targeted maternal gene in early embryogenesis, rescues the system[16,47]. Only progeny who have successfully received the MEDEA system and antidote from the MEDEA-bearing mother survive, whereas non-MEDEA-bearing progeny lacking the antidote perish during embryogenesis[47]. Previous use of the MEDEA system has delineated a high initial introduction rate (25%) requirement, which can pose technical issues for certain projects[32]. On the other hand, the HEG system takes advantage of the homing endonuclease gene, a selfish genetic element indigenous to microbes which recognizes a unique 18-30bp sequence in the genome[48]. The gene inserts into the center of the recognition sequence and protects the chromosome from being cut. The non-HEG homologous chromosome gets cut and utilizes the HEG-chromosome as a template for repair, thereby producing a homozygous individual. HEG systems can be utilized to identify a portion of the target mosquito gene and may be inserted into the middle of its own recognition sequence. HEG can be utilized to knockout genes needed for mosquito survival, thereby reducing vector density. It can also be engineered to knockout genes that promote the development of *P. falciparum*, ultimately reducing vector competence[47].

Mosquito Vector Control – Gut Microbiota

Transgenesis typically poses significant problems, such as altering *Anopheles* fitness such that they cannot compete with the wild vector population. Therefore, current research efforts seek to identify the extent to which coopting the mosquito gut microbiota can attenuate *Plasmodium* transmission. Through the manipulation of the gut microbiota, it is still possible to identify important microbial strains, which could either indirectly or directly halt the sexual cycle.

The gut microbiota is of particular importance when elucidating pathogen-insect interactions since it is

the primary site that is exposed to the parasite. It is known to provide mosquitos with many benefits, such as assisting in digestion to provide nutritional supplements, affecting host immune responses, and providing colonization resistance towards pathogens by competing for resources (niche competition), or secreting antimicrobial molecules[8,49,50]. Past studies have demonstrated increased susceptibility of antibiotic-treated *Anopheles* towards developing *Plasmodium* colonization when compared to mosquitoes with a functional gut microbiota[51]. Therefore, the gut microbiota plays a critical role in attenuating the progression of the *Plasmodium* sexual cycle and serves as a potent intervention site.

Mosquito exposure to microbes occurs throughout their development, but they initially acquire microbes from the mother's genitalia and from larval and pupal breeding sites[16]. The environmental conditions from which a mosquito develops contributes to vast differences seen amongst various *Anopheles* species[8,52]. Throughout the mosquito's lifespan, a core microbiota is established, which is continuously altered after a blood meal ingestion. These microbial fluctuations activate immune responses such as the immune deficiency (Imd) pathway, in order to limit the parasitic infection[16]. The Imd pathway assists in maintaining a basal level of immunity and is activated upon the detection of peptidoglycan, a core component of the bacterial cell wall[53]. However, bacteria can also directly affect the development of the parasite. A past study isolated *Enterobacter sp.* Esp_Z from the midgut of wild *Anopheles arabiensis* and identified its ability to impair the parasite's sexual cycle[55]. The production of reactive oxygen species by Esp_Z was implicated in its mode of action, which involves interrupting ookinete invasion of the midgut epithelium.

An alternative approach that researchers utilize to influence the midgut microbiota of *Anopheles* mosquitoes is known as paratransgenesis, whereby symbiotic bacteria are genetically engineered to express effector molecules. These engineered microbes are then fed to mosquitoes during their blood meal[55]. Bacteria that are used for the paratransgenic approach must meet three requirements: the effector molecule must function with the desirable effect; there must be a mechanism to secrete or display the effector molecule on the bacterial surface; and the bacteria must survive for a considerable duration to maintain a high concentration of the effector molecule to achieve the desired consequence[55].

Current research efforts seek to bridge the link between the mosquito midgut microbiota and malaria attenuation by utilizing bioinformatics approaches to elucidate the production potential of indigenous gut microbes. An extensive literature search has been performed to identify the various genera, species and strains present in the *Anopheles* midgut microbiota. A particularly useful paper written by Minard *et al.* (2013) highlights dominant genera present in the midgut microbiota of *Aedes albopictus* and *Anopheles stephensi* (Table 1)[56]. Other papers have also identified genera predominant in the midgut microbiota[13,52,57]. To identify production potential, two approaches have been utilized: in the first approach, full genome sequences (FASTA format) were downloaded and fed through the AntiSMASH algorithm, to identify strains encoding a high-volume of biosynthetic gene clusters (BGCs)[59]. These putative biosynthetic gene clusters were verified utilizing a pull-in data set (Quince), developed by the Magarvey lab. These talented strains are currently undergoing production media panels to stimulate BGC's for natural product discovery.

Table 1: A curated list of select bacterial genera which can be found in *Aedes albopictus* and *Anopheles stephensi*. The identification of these genera have informed strain selection for media panels[53].

***Aedes albopictus* Bacterial Genera**	***Anopheles stephensi* Bacterial Genera**
Staphylococcus	*Xenorhabdus*
Bacillus	*Bacillus*
Planococcus	*Acineobacter*
Lactococcus	*Photorhabdus*
Paenibacillus	*Paenibacillus*
Arsenicicoccus	*Bordetella*
Terrabacter	*Leminorella*
Dermacoccus	*Agrobacterium*
Micrococcus	*Comamonas*
Arthrobacter	*Alcaligenes*
Kocuria	*Achromobacter*
Methylobacterium	*Chryseobacterium*
Acetobacter	*Elizabethkingia*
Skermanella	*Flavobacterium*
Pantoea	*Pantoea*
Citrobacter	*Klebsiella*
Serratia	*Serratia*
Aeromonas	*Herbaspirillum*
Pseudomonas	*Myroides*

Future Directions

The evolution of sequencing strategies can help identify differences amongst the gut microbiota of various *Anopheles* mosquitoes in a species-specific manner. This strategy can assist in elucidating certain bacterial strains that may prevent certain mosquitoes from carrying *P. falciparum.* These sequencing approaches can also help determine the microbiota of various mosquito regions such as the salivary gland and hemocoel in better depth, to further establish their potential as intervention sites.

An interesting area of research that is currently under investigation includes the impact of the vertebral skin microbiota in promoting the transmission of *P. falciparum.* Previous studies have suggested that an individual's skin microbiota composition affects the vertebral host's degree of attractiveness[59]. In fact, researchers have discovered that *Plasmodium*-infected hosts with high gametocytic loads possess altered skin microbiota that further increases their attractiveness towards *Anopheles* mosquitoes, thereby allowing for the propagation of the parasite[12]. These studies suggest host odour can be targeted to attenuate malarial transmission, and further research efforts can determine methodology needed to alter mosquito olfactory cues to prevent them from blood feeding on infected vertebral hosts[10].

Recently, Contreras *et al.* (2019) identified a neurotoxin, PMP1 (paraclostridial mosquitocidal protein 1) produced by *Paraclostridium bifermentans* strains that are capable of paralyzing *Anopheles* mosquitoes via syntaxin cleavage, resulting in the disruption SNARE-mediated exocytosis[60]. This finding can be used in conjunction with the strategies stated above, whereby artificial blood meals containing the PMP1 neurotoxin are coated with bacteria known to attract *Anopheles* mosquitoes; these can be distributed across homes in highly endemic areas, to be utilized as prevention strategies similar to IRS and insecticide-treated bed nets.

Acknowledgements

This work was completed as part of the requirements of BIOCHEM 4T15 Fall 2019, at McMaster University.

References

1. World Health Organization. (2019) Malaria. *WHO*.
2. Miller, L. H., Ackerman, H. C., Su, X. Z., and Wellems, T. E. (2013) Malaria biology and disease pathogenesis: Insights for new treatments. *Nat. Med. 19*, 156–167.
3. Cowman, A. F., Healer, J., Marapana, D., and Marsh, K. (2016) Malaria: Biology and Disease. *Cell 167*, 610–624.
4. Rasti, N., Wahlgren, M., and Chen, Q. (2004) Molecular aspects of malaria pathogenesis. *FEMS Immunol. Med. Microbiol. 41*, 9–26.
5. Prevention, C. for D. C. and Malaria.
6. Wassmer, S. C., Emile, G., and Grau, R. (2017) Severe malaria: what's new on the pathogenesis front? Graphical abstract HHS Public Access. *Int J Parasitol 47*, 145–152.
7. Fang, W., Vega-Rodriguez, J., Ghosh, A. K., Jacobs-Lorena, M., Kang, A., and St. Leger, R. J. (2011) Development of Transgenic Fungi That Kill Human Malaria Parasites in Mosquitoes. *Science (80-.). 331*, 1074–1077.
8. Dennison, N. J., Jupatanakul, N., and Dimopoulos, G. (2014) The mosquito microbiota influences vector competence for human pathogens. *Curr. Opin. Insect Sci. 3*, 6–13.
9. Wunderlich, J., Rohrbach, P., and Dalton, J. P. (2012) The malaria digestive vacuole. *Front. Biosci. - Sch. 4 S*, 1424–1448.
10. Kalappa, D. M., Subramani, P. A., Basavanna, S. K., Ghosh, S. K., Sundaramurthy, V., Uragayala, S., Tiwari, S., Anvikar, A. R., and Valecha, N. (2018) Influence of midgut microbiota in Anopheles stephensi on Plasmodium berghei infections. *Malar. J. 17*, 1-8.
11. Kumar, H., and Tolia, N. H. (2019) Getting in: The structural biology of malaria invasion. *PLOS Pathog. 15*, e1007943.
12. Busula, A. O., Verhulst, N. O., Bousema, T., Takken, W., and de Boer, J. G. (2017) Mechanisms of Plasmodium-Enhanced Attraction of Mosquito Vectors. *Trends Parasitol. 33*, 961–973.
13. Tchioffo, M. T., Boissière, A., Churcher, T. S., Abate, L., Gimonneau, G., Nsango, S. E., Awono-Ambéné, P. H., Christen, R., Berry, A., and Morlais, I. (2013) Modulation of malaria infection in Anopheles gambiae mosquitoes exposed to natural midgut bacteria. *PLoS One 8*, 4–12.
14. Romoli, O., and Gendrin, M. (2018, March 20) The tripartite interactions between the mosquito, its microbiota and Plasmodium. *Parasites and Vectors*. BioMed Central Ltd.
15. Bando, H., Okado, K., Guelbeogo, W. M., Badolo, A., Aonuma, H., Nelson, B., Fukumoto, S., Xuan, X., Sagnon, N., and Kanuka, H. (2013) Intra-specific diversity of Serratia marcescens in Anopheles mosquito midgut defines Plasmodium transmission capacity. *Sci. Rep. 3*, 1–9.

16. Wang, S., and Jacobs-Lorena, M. (2013) Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends Biotechnol. 31*, 185–193.
17. Dinglasan, R. R., Devenport, M., Florens, L., Johnson, J. R., McHugh, C. A., Donnelly-Doman, M., Carucci, D. J., Yates, J. R., and Jacobs-Lorena, M. (2009) The Anopheles gambiae adult midgut peritrophic matrix proteome. *Insect Biochem. Mol. Biol. 39*, 125–134.
18. Saraiva, R. G. (2018) Kosakonia & chromobacterium vs. malaria & dengue – Insights into antipathogenic strategies of mosquito midgut bacteria. *John Hopkins University.*
19. Enayati, A., and Hemingway, J. (2010) Malaria Management: Past, Present, and Future. *Annu. Rev. Entomol. 55*, 569–591.
20. OMS. (2015) Indoor Residual Spraying. An Operational Manual for Indoor Residual Spraying (IRS) for Malaria Transmission Control and Elimination. *World Heal. Organ.* 1–125.
21. Achan, J., Talisuna, A.O. , Erhart, A. , Yeka, A., Tibenderana, J.K., Baliraine, F.N., Rosenthal, P.J. and D'Alessandro U. (2011) Quinine, an old anti-malarial drug in a modern world: Role in the treatment of malaria. *Malar. J. 10*, 1–12.
22. Wells, T. N. C., Alonso, P. L., and Gutteridge, W. E. (2009) New medicines to improve control and contribute to the eradication of malaria. *Nat. Rev. Drug Discov. 8*, 879–891.
23. Wellems, T. E., and Plowe, C. V. Chloroquine-Resistant Malaria 770–776.
24. Eastman, R. T., and Fidock, D. A. (2009) Artemisinin-based combination therapies: A vital tool in efforts to eliminate malaria. *Nat. Rev. Microbiol. 7*, 864–874.
25. Payne, D. (1987) Spread of chloroquine resistance in Plasmodium falciparum. *Parasitol. Today 3*, 241–6.
26. Juge, N., Moriyama, S., Miyaji, T., Kawakami, M., Iwai, H., Fukui, T., Nelson, N., Omote, H., and Moriyama, Y. (2015) Plasmodium falciparum chloroquine resistance transporter is a H + -coupled polyspecific nutrient and drug exporter. *Proc. Natl. Acad. Sci. U. S. A. 112*, 3356–3361.
27. Martin, R. E., Marchetti, R. V., Cowan, A. I., Howitt, S. M., Bröer, S., and Kirk, K. (2009) Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science 325*, 1680–1682.
28. Luo, X.-D., and Shen, C.-C. (1987) The chemistry, pharmacology and clinical applications from qinghaosu (Artemisinin) and its derivatives. *Med. Res. Rev. 7*, 29–52.
29. Jiang, J. B., Guo, X. B., Li, G. Q., Cheung Kong, Y., and Arnold, K. (1982) Antimalarial Activity of Mefloquine and Qinghaosu. *Lancet 320*, 285–288.
30. Woodrow, C. J., and Krishna, S. (2006) Antimalarial drugs: Recent advances in molecular determinants of resistance and their clinical significance. *Cell. Mol. Life Sci. 63*, 1586–1596.
31. World Health Organization. (2017) Artemisinin and artemisinin-based combination therapy resistance. *WHO* 1–11.
32. Hemingway, J., Shretta, R., Wells, T. N. C., Bell, D., Djimdé, A. A., Achee, N., and Qi, G. (2016) Tools and Strategies for Malaria Control and Elimination: What Do We Need to Achieve a Grand Convergence in Malaria? *PLoS Biol. 14*, 1–14.
33. Hill, A. V. S. (2006) Pre-erythrocytic malaria vaccines: Towards greater efficacy. *Nat. Rev. Immunol. 6*, 21–32.
34. The malERA Consultative Group on Vaccines. (2011) A research agenda for malaria eradication: Vaccines. *PLoS Med. 8*.
35. Nikolaeva, D., Draper, S. J., and Biswas, S. (2015) Toward the development of effective transmission-blocking vaccines for malaria. *Expert Rev. Vaccines 14*, 653–680.
36. Birkett, A. J. (2010) PATH malaria vaccine initiative (MVI): Perspectives on the status of malaria vaccine development. *Hum. Vaccin. 6*, 139–145.
37. Milicic, A., Rollier, C. S., Tang, C. K., Longley, R., Hill, A. V. S., and Reyes-Sandoval, A. (2017) Adjuvanting a viral vectored vaccine against pre-erythrocytic malaria. *Sci. Rep. 7*, 1–12.
38. Good, M. F. (2001) Towards a blood-stage vaccine for malaria: Are we following all the leads? *Nat. Rev. Immunol. 1*, 117–125.
39. Rener, B. Y. J., Graves, P. M., Carter, R., Williams, J. L., and Burkot, T. R. (1983) Target Antigens of Transmission-Blocking Immunity on Gametes of Plasmodium Falciparum *158*, 976–981.
40. Kokoza, V., Ahmed, A., Shin, S. W., Okafor, N., Zou, Z., and Raikhel, A. S. (2010) Blocking of Plasmodium transmission by cooperative action of Cecropin a and Defensin a in transgenic Aedes aegypti mosquitoes. *Proc. Natl. Acad. Sci. U. S. A. 107*, 8111–8116.
41. Vizioli, J., Bulet, P., Hoffmann, J. A., Kafatos, F. C., Müller, H. M., and Dimopoulos, G. (2001) Gambicin: A novel immune responsive antimicrobial peptide from the malaria vector Anopheles gambiae. *Proc. Natl. Acad. Sci. U. S.*

A. 98, 12630–12635.
42. Ghosh, A. K., Coppens, I., Gårdsvoll, H., Ploug, M., and Jacobs-Lorena, M. (2011) Plasmodium ookinetes coopt mammalian plasminogen to invade the mosquito midgut. *Proc. Natl. Acad. Sci. U. S. A. 108*, 17153–17158.
43. Ghosh, A. K., Ribolla, P. E. M., and Jacobs-Lorena, M. (2001) Targeting Plasmodium ligands on mosquito salivary glands and midgut with a phage display peptide library. *Proc. Natl. Acad. Sci. U. S. A. 98*, 13278–13281.
44. Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A., and Jacobs-Lorena, M. (2002) Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature 417*, 452–455.
45. McArthur, C. C., Meredith, J. M., and Eggleston, P. (2014) Transgenic Anopheles gambiae expressing an antimalarial peptide suffer no significant fitness cost. *PLoS One 9*.
46. Gantz, V. M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V. M., Bier, E., and James, A. A. (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proc. Natl. Acad. Sci. U. S. A. 112*, E6736–E6743.
47. Buchman, A., Marshall, J. M., Ostrovski, D., Yang, T., and Akbari, O. S. (2018) Synthetically engineered Medea gene drive system in the worldwide crop pest Drosophila suzukii. *Proc. Natl. Acad. Sci. U. S. A. 115*, 4725–4730.
48. Deredec, A., Godfray, H. C. J., and Burt, A. (2011) Requirements for effective malaria control with homing endonuclease genes. *Proc. Natl. Acad. Sci. U. S. A. 108*.
49. Cirimotich, C. M., Ramirez, J. L., and Dimopoulos, G. (2011) Native microbiota shape insect vector competence for human pathogens. *Cell Host Microbe 10*, 307–310.
50. Weiss, B., and Aksoy, S. (2011) Microbiome influences on insect host vector competence. *Trends Parasitol. 27*, 514–522.
51. Dong, Y., Manfredini, F., and Dimopoulos, G. (2009) Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog. 5*.
52. Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., Shahbazkia, H. R., Awono-Ambene, P. H., Levashina, E. A., Christen, R., and Morlais, I. (2012) Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium falciparum infection. *PLoS Pathog. 8*, 1–12.
53. Habtewold, T., Groom, Z., and Christophides, G. K. (2017) Immune resistance and tolerance strategies in malaria vector and non-vector mosquitoes. *Parasites and Vectors 10*, 1–12.
54. Cirimotich, C. M., Dong, Y., Clayton, A. M., Sandiford, S. L., Souza-Neto, J. A., Mulenga, M., and Dimopoulos, G. (2011) Natural Microbe-Mediated Refractoriness to Plasmodium Infection in Anopheles gambiae *332*.
55. Wilke, A. B. B., and Marrelli, M. T. (2015) Paratransgenesis: A promising new strategy for mosquito vector control. *Parasites and Vectors 8*, 1–9.
56. Minard, G., Mavingui, P., and Moro, C. V. (2013) Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasites and Vectors 6*, 1.
57. Gimonneau, G., Tchioffo, M. T., Abate, L., Boissière, A., Awono-ambéné, P. H., Nsango, S. E., Christen, R., and Morlais, I. (2014) Composition of Anopheles coluzzii and Anopheles gambiae microbiota from larval to adult stages. *Infect. Genet. Evol. 28*, 715–724.
58. Medema, M. H., Blin, K., Cimermancic, P., De Jager, V., Zakrzewski, P., Fischbach, M. A., Weber, T., Takano, E., and Breitling, R. (2011) AntiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res. 39*, 339–346.
59. Verhulst, N. O., Qiu, Y. T., Beijleveld, H., Maliepaard, C., Knights, D., Schulz, S., Berg-Lyons, D., Lauber, C. L., Verduijn, W., Haasnoot, G. W., Mumm, R., Bouwmeester, H. J., Claas, F. H. J., Dicke, M., van Loon, J. J. A., Takken, W., Knight, R., and Smallegange, R. C. (2011) Composition of human skin microbiota affects attractiveness to malaria mosquitoes. *PLoS One 6*.
60. Contreras, E., Masuyer, G., Qureshi, N., Chawla, S., Dhillon, H. S., Lee, H. L., Chen, J., Stenmark, P., and Gill, S. S. (2019) A neurotoxin that specifically targets Anopheles mosquitoes. *Nat. Commun. 10*, 2869.
61. Brown, A. E., and Catteruccia, F. (2006) Toward silencing the burden of malaria: progress and prospects for RNAi-based approaches. *Biotechniques Suppl*, 38–44.
62. Wells, T. N. C., Van Huijsduijnen, R. H., and Van Voorhis, W. C. (2015) Malaria medicines: A glass half full? *Nat. Rev. Drug Discov. 14*, 424–442.
63. Center for Disease Control and Prevention. (2019) Indoor Residual Spraying.
64. Prevention, C. for D. C. and. (2019) Insecticide-Treated Bed Nets.
65. Aoki, S., Shteyn, K., and Marien, R. (2017) BioRender. Toronto.