School of Pharmacy and Biomedical Sciences

Investigating the Relationship Between Statins and Bacterial Skin Infections

Hean Teik Humphrey Ko

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

November 2019

DECLARATION

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated May 2015. The proposed research study received human research ethics approval from the South Metropolitan Health Service Human Research Ethics Committee (Approval Number: 12/285), the Department of Veterans' Affairs Human Research Ethics Committee (Approval Number: E014/003), and reciprocal human research ethics approval from the Curtin University Human Research Ethics Committee (Approval Number: HR155/2015).

Signature: _____

Hean Teik Humphrey Ko

Date: 22nd November 2019

ABSTRACT

Background: The World Health Organization has warned that antimicrobial resistance (AMR) may herald a post-antibiotic era whereby last-line antibiotics may become ineffective. This crisis is compounded by the lack of novel antibiotics for over a decade. Finding new uses for existing drugs (drug repurposing) confers advantages such as significant financial savings, potential to impede AMR, and the prospect of connecting laboratory research with clinical practice research (translational research).

Skin and soft tissue infections (SSTIs) are common infections that consume significant healthcare resources and require frequent antibiotic administration, potentially contributing to AMR. Amongst a myriad of risk factors for SSTIs, previous episodes of SSTIs and diabetes (which predisposes patients to *Staphylococcus aureus* colonisation and infections) increase the incidence of recurrent SSTIs, developing a vicious cycle of infections.

The search for an effective solution led to the research question of whether statins, a class of medicines extensively prescribed globally to prevent cardiovascular diseases, could be repurposed as potential novel adjuvants/treatments for bacterial SSTIs, thus potentially impeding AMR and saving substantial healthcare resources.

Objectives: This research on the association between statins and bacterial SSTIs sought to address the following aims in order to answer the overarching research question of whether statins may be repurposed as novel agents for bacterial SSTIs:

1. To evaluate the effect of statins on AMR based on current literature and identify if there was sufficient evidence to support statins as novel antimicrobial agents (Chapter Two).
2. To determine the antibacterial activity of statins against selected bacterial pathogens implicated in SSTIs, ascertain if the activity was bacteriostatic or bactericidal, and postulate a plausible mechanism of action (Chapter Three).
3. To determine the direct relationship between statins and SSTIs, along with the association between statins and diabetes, a risk factor for *S. aureus*-related

SSTIs which predisposes patients to recurrent SSTIs (Chapters Four and Five).

Methods: The relationship between statins and bacterial SSTIs was studied by adopting a translational research framework, whereby laboratory evidence was reconciled with clinical evidence to address whether statins may be repurposed as novel therapeutic agents for SSTIs. A comprehensive literature review was performed in accordance with the requirements of a systematic review using the keywords "statin" or "statins" combined with "minimum inhibitory concentration" (MIC) in six databases. Further analysis was performed to evaluate the impact of statins on bacteria, humans, and the environment (Objective 1).

Laboratory experiments involved testing the direct antibacterial effects of all clinically approved statins (atorvastatin [ATV], fluvastatin [FLV], lovastatin [LVS], pitavastatin [PTV], pravastatin [PRV], rosuvastatin [RSV], and simvastatin [SMV]), together with three selected metabolites (LVS hydroxy acid sodium [LVS-OH acid], PTV-lactone, and SMV hydroxy acid sodium [SMV-OH acid]) against bacterial skin pathogens *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens* using broth microdilution methods according to the guidelines stipulated by the Clinical Laboratory Standards Institute. A structure-activity relationship analysis was also performed by reconciling the chemical structure of statins and the selected metabolites with their respective MICs to postulate a plausible mechanism of antibacterial activity (Objective 2).

A sequence symmetry analysis (SSA) was performed on outpatient prescription claims from the Australian Department of Veterans' Affairs (DVA) to determine the interrelationships between statins, diabetes, and skin infections (Objective 3). A retrospective matched case-control study (SSTI cases, n = 165; controls without SSTIs, n = 165) was conducted on inpatients admitted in the Medical Ward of Rockingham General Hospital, Western Australia. The primary analysis of this study aimed to determine: (i) the association between statin use and the risk of SSTIs and (ii) whether the use of statins was associated with improved clinical outcomes. A secondary analysis on the subgroup of patients with an SSTI infection determined the

association between statin use and: (i) the incidence of diabetes and (ii) clinical outcome indicators (Objective 3).

Results: The 16 studies used in the literature review showed that current evidence better supports statins as AMR breakers, with SMV demonstrating the most promise as a novel adjuvant antibiotic (Objective 1). However, further analysis within a statin-bacteria-human-environment continuum also raised the possibility of statins contributing to AMR.

Laboratory experiments demonstrated that SMV (MIC = 64 μg/mL), PTV-lactone (MIC = 128 μg/mL), ATV, and FLV ($MIC_{[ATV]} = MIC_{[FLV]}$ = 256 μg/mL) exerted bacteriostatic effects against *S. aureus*. None of the statins or metabolites exerted antibacterial effects against *E. coli*, *P. aeruginosa*, or *S. marcescens.* Through a structure-activity relationship analysis, it was postulated that statins' antibacterial action may involve statins binding with alanine residues of teichoic acids present on Gram-positive bacterial cell surfaces. This may occur via interactions involving the combination of a hydrophobic statin ring system, a lactone ring moiety, and a *gem*-dimethyl moiety or a cyclopropyl ring (Objective 2).

From the SSA on DVA prescription data, statins were associated with: (i) significantly increased risks of SSTIs, (ii) significant increased risks of diabetes, and (iii) diabetic patients had significantly increased risk of SSTIs. Diabetic and non-diabetic statin users had significantly increased risks of SSTIs, while the influence from socio-economic status was not significant for each of the three relationships (Objective 3). The primary analysis from the case-control study on inpatients demonstrated (i) the use of ATV, PRV, and SMV was not significantly associated with SSTIs, along with (ii) no significant differences in clinical outcomes between stain users and non-statin users. In the secondary analysis on inpatients with an SSTI, (i) the use of ATV was associated with a significantly increased risk of diabetes (RR = 2.854, p = 0.001) and (ii) no significant differences in clinical outcomes between statin users and non-statin users (Objective 3).

Conclusions and Recommendations: By reconciling laboratory evidence with clinical evidence, it is unlikely that statins which are associated with significant risk

of diabetes (ATV, FLV, LVS, PRV, RSV, and SMV) may serve as novel therapeutic agents for SSTIs. Statins may increase the risk of SSTIs through a direct mechanism (reduction of innate immunity) or through an indirect mechanism (increasing the risk of diabetes, in turn a risk factor for SSTIs). The combined possibility of systemic absorption, lack of antibacterial activity against pathogens causing severe SSTIs, and risk of statin contribution to AMR collectively mitigate laboratory evidence for the use of statins as topical novel therapeutic agents. Further research on PTV in a country where it is registered for clinical use might corroborate if it is the only statin with potential for repurposing as a novel therapeutic agent for SSTIs due to its favourable effects on diabetes and obesity.

Of greater concern however, this research unravelled the ominous possibility that extensive use of statins globally could contribute to AMR via selective pressures or co-selection for resistance, which warrants further investigation beyond the scope of this thesis. It is hoped that the postulated mechanism of statins' antibacterial action and suggested common areas of research in the human gut microbiome and PXRs, amongst other contributions in this thesis, might support and invoke further research in the search for other novel SSTI treatments, in tandem with addressing statins' influence on AMR.

ACKNOWLEDGEMENTS

As I draw closer towards my hope of being a practising academic pharmacist, I am truly grateful to the following people who have and continue to shower me with immense Kindness and Love in my journey:

Prof Jeffery Hughes, for guiding, encouraging, and organising opportunities for me to work as a hospital pharmacist and sessional tutor even before my PhD course.

Dr Ricky Lareu, for motivating and mentoring me in my research, furthering my sessional opportunities, and training me to facilitate student learning.

Dr Brett Dix, for coaching me in the finer aspects of laboratory techniques and advice in establishing the foundation for my lab work.

Dr Richard Parsons, for tutoring me through statistics and inspiring me to further engage in this essential component of research.

Dr Nicole Pratt (University of South Australia) and her co-authors,[1] who obligingly shared their sequence symmetry analysis SAS program codes.

The authorities of the Australian Government Research Training Program, for kindly funding my research.

My colleagues (especially Ms Angela Samec) at the School of Pharmacy and Biomedical Sciences in Curtin University and fellow PhD mates (especially Ms Martha Mungkaje) at the Curtin Health Innovation Research Institute Biosciences Research Precinct, for supporting me through technical trouble shooting and their amiable companionship.

The staff and ethics committees of the Australian Department of Veterans' Affairs and Western Australian South Metropolitan Health Services, for patiently compiling and granting me access to the raw data for my research.

Mr Graham Stannard, for personifying empathy - I will pay it forward.

Mr Wu Tuck Seng, for shepherding me to be the best pharmacist I can be.

My communities from SJI, NJC, NUS, MM, NUH, TP, SMC, SC, RPH, SCGH, and RGH - acronyms are just institutions, but bonds formed therein remain always.

Ian and Aunty Ngoh, for taking care of me since my first day in Perth.

My family at home in Singapore, especially my mother (Suan Suan) and sister (Katherine), who have loved me all my life and moulded all that is good in me.

LIST OF PUBLICATIONS AND PRESENTATIONS

The work leading to this thesis has contributed to the following publications and presentations:

Publications on work which form this thesis

Peer-Reviewed Manuscripts

- **Ko H**, Lareu RR, Dix BR, Hughes JD, Parsons RW. A sequence symmetry analysis of the interrelationships between statins, diabetes, and skin infections. Br J Clin Pharmacol. 2019; 85(11):2559-2567. doi:10.1111/bcp.14077.
- **Ko H**, Lareu RR, Dix BR, Hughes JD. *In vitro* antibacterial effects of statins against bacterial pathogens causing skin infections. Eur J Clin Microbiol Infect Dis. 2018; 37(6):1125-1135. doi:10.1007/s10096-018-3227-5.
- **Ko H**, Lareu RR, Dix BR, Hughes JD. Statins: antimicrobial resistance breakers or makers? PeerJ. 2017;5:e3952. doi:10.7717/peerj.3952.

Publications on reflections of other researchers' work related to this thesis

Letters to the Editor

- **Ko H**, Lareu RR, Dix BR, Hughes JD. Effect of statins on sepsis outcome in a population-based cohort study. Chest. 2018;154:718-9. doi:10.1016/j.chest.2018.04.046.
- **Ko H**, Lareu RR, Dix BR, Hughes JD. Statin use associated with a decreased risk of community-acquired *Staphylococcus aureus* bacteremia. Mayo Clinic Proceedings. 2018;93(4):541-2. doi:10.1016/j.mayocp.2017.12.024.

Presentations

- **Ko H**, Lareu RR, Dix BR, Hughes JD, Parsons R. Prescription of statins: A life trajectory to diabetes mellitus? Poster Presentation at the Science on the Swan Conference held from 1st to 3rd May 2018 in Fremantle, Perth, Australia.
- **Ko H,** Lareu R, Dix B, Hughes J. Using prescription sequence symmetry analysis to determine if statins cause skin infections and/or diabetes. Oral presentation at the Mark Liveris Health Sciences Research Student Seminar held on 1st September 2016 in Curtin University, Perth, Australia.

- **Ko H,** Lareu R, Dix B, Hughes J. Statins – antimicrobial effects and resistance. Poster presentation at the Australian Society for Antimicrobials' 17th Annual Scientific Meeting – Antimicrobials 2016 held from 25th to 27th February 2016 in Melbourne Exhibition Centre, Melbourne, Australia.
- **Ko H,** Lareu R, Dix B, Hughes J. My anti-cholesterol medicine is anti-bacterial too! Oral presentation at the Mark Liveris Health Sciences Research Student Seminar held on 3rd September 2015 in Curtin University, Perth, Australia.

TABLE OF CONTENTS

LIST OF ACRONYMS AND ABBREVIATIONS

ABSSSI:	Acute bacterial skin and skin structure infection
ACEI:	Angiotensin-converting enzyme inhibitor
AMR:	Antimicrobial resistance
ANOVA:	Analysis of variance
ARB:	Angiotensin II receptor blocker
ASR:	Adjusted sequence ratio
ATCC:	American Type Culture Collection
ATV:	Atorvastatin
CAMHB:	Cation-adjusted Mueller Hinton broth
CFU:	Colony-forming unit
CI:	Confidence interval
CINAHL:	Cumulative Index to Nursing and Allied Health Literature
CLSI:	Clinical and Laboratory Standards Institute
COPD:	Chronic obstructive pulmonary disease
CSR:	Crude sequence ratio
DMSO:	Dimethyl sulfoxide
DVA:	Department of Veterans' Affairs
ESKAPE:	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species
FDA:	Food and Drug Administration
FLV:	Fluvastatin
FPP:	Farnesyl pyrophosphate
FXR:	Farnesoid X receptor
GC:	Growth control
GCR:	Glucocorticoid receptor
HIV:	Human immunodeficiency virus
HMG-CoA:	3-hydroxy-3-methylglutaryl Coenzyme A
ICD-10:	International Statistical Classification of Diseases and Related Health Problems, 10th revision
IRSAD:	Index of Relative Socio-economic Advantage and Disadvantage
LDL:	Low-density lipoprotein
LDL-C:	Low-density lipoprotein cholesterol

LVS: Lovastatin

LVS-OH: Lovastatin hydroxy

MIC: Minimum inhibitory concentration

MRCoNS: Methicillin-resistant coagulase negative *Staphylococcus aureus*

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSSA: Methicillin-susceptible *Staphylococcus aureus*

NCCLS: National Committee for Clinical Laboratory Standards

NET: Neutrophil extracellular trap

ns: Not significant

NSR: Null-effect sequence ratio

NT: Not tested

OD625: Optical density at wavelength 625 nm

OR: Odds ratio

PAMP: Pathogen associated molecular pattern

PPARγ: Peroxisome proliferator-activated receptor gamma

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PRV: Pravastatin

PTV: Pitavastatin

PXR: Pregnane X receptor

RR: Relative risk

RSV: Rosuvastatin

SC: Sterility control

SD: Standard deviation

SMV: Simvastatin

SMV-OH: Simvastatin hydroxy

SSA: Sequence symmetry analysis

SSTI: Skin and soft tissue infection

Th1: T helper cell type 1

Th17: T helper cell type 17

TNTC: Too numerous to count

Treg: T regulatory cells

VDR: Vitamin D receptor

VISA: Vancomycin-intermediate *Staphylococcus aureus*

VRE: Vancomycin-resistant *Enterococci*

VRSA: Vancomycin-resistant *Staphylococcus aureus*

LIST OF FIGURES

LIST OF TABLES

PREFACE

The primary investigator thanks all readers for taking the time to review this thesis, which is comprised of published and unpublished research material, conforms to the Vancouver referencing style, and has been written in British English. It details the hypothesis that statins, an extensively prescribed class of medicines for reducing the risk of cardiovascular diseases, may potentially be repurposed to serve as novel antibacterial agents to treat bacterial skin infections. In doing so, the need to use vital last-line antibiotics in this era of antimicrobial resistance would be reduced and significant healthcare resources could potentially be saved.

Briefly, the thesis has been organised as follows:

Chapter One:	Introduction
Chapter Two:	Literature Review on Statins' Antibacterial Effects
Chapter Three:	Laboratory Evidence (Antibacterial Effects Against Skin Pathogens)
Chapter Four:	Ambulatory Care Evidence (Sequence Symmetry Analysis)
Chapter Five:	Hospital Care Evidence (Case-Control Study)
Chapter Six:	Discussion of Accumulated Evidence
Chapter Seven:	Conclusions and Recommendations

Peer-reviewed publications from this research were derived from a comprehensive review in accordance with the requirements of a systematic review (Chapter Two), and results of laboratory experiments conducted at the Curtin Health Innovation Research Institute (Chapter Three). Further, a manuscript utilising sequence symmetry analysis of prescription data from the Australian Department of Veterans' Affairs has been accepted for publication (Chapter Four). Lastly, a case-control study of hospitalised patients was conducted in Rockingham General Hospital, where the primary investigator works as a pharmacist. This study has recently been concluded and the unpublished findings are presented in Chapter Five of the thesis.

In summary, this research found little evidence supporting the original hypothesis of statins serving as novel antibacterial agents. Rather, the accumulated evidence suggested an ominous possibility that statins may be associated with antimicrobial resistance instead. This is an important finding given the widespread global use of statins and as such, warrants further investigation beyond the scope of this thesis.

CHAPTER ONE

1. Introduction

1.1 Research Overview

As the world approaches a post-antibiotic era whereby last-line antibiotics may become ineffective due to antimicrobial resistance (AMR),[2] compounded by the drought of novel antibiotics for over a decade,[3] there exists a real threat of increased mortality from common infections and minor injuries which were once easily treated. The process of finding new uses for old drugs (drug repurposing or repositioning) has been shown to be a viable research area for bacterial infections,[3] with advantages such as huge financial savings via established essential drug properties and safety information gleaned from previous clinical trials,[4] the potential to impede AMR by serving as "AMR breakers",[5] as well as the prospect of bridging basic scientific research with applied research in clinical practice (translational research).[6]

This introduction (Chapter One) expounds on the research problems (in red boxes; Figure 1-1), whereby skin and soft tissue infections (SSTIs) are common infections that consume significant healthcare resources and require frequent antibiotic administration, potentially contributing to AMR. The search for an effective solution led to the research question of whether statins, an extensively prescribed class of medicines to reduce cholesterol,[7] could be repurposed as potential novel adjuvants/treatments for bacterial SSTIs via studying the relationship between statins and bacterial skin infections.

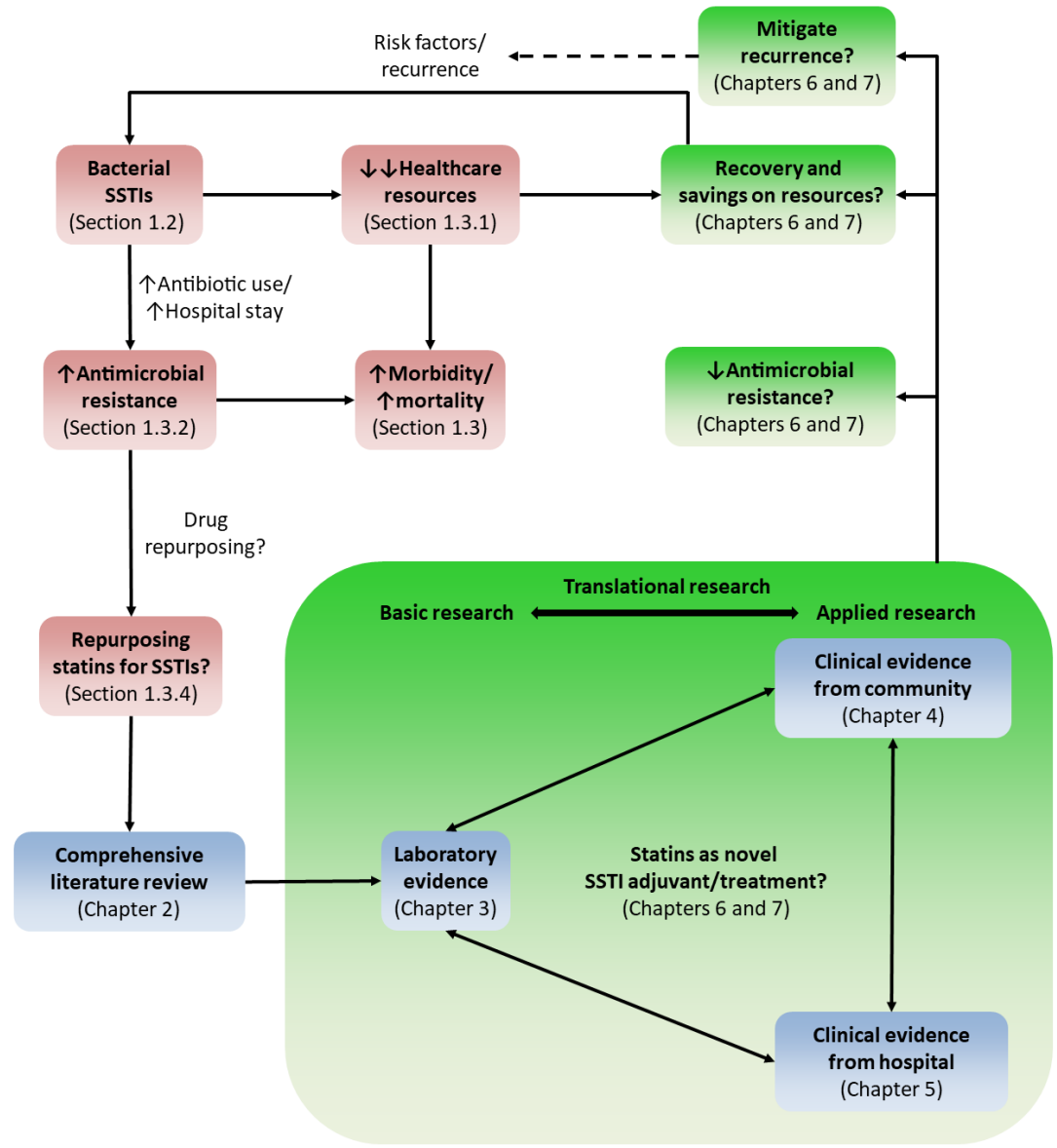

Figure 1-1: Overview of thesis research.
Flowchart interweaving the research problems and question (in red boxes), specific research projects (in blue boxes), and overall research objectives (in green boxes) to evaluate the hypothesis that statins may potentially be repurposed to serve as novel antibacterial agents to treat bacterial SSTIs and mitigate SSTI recurrence, thereby conceivably reducing AMR and saving considerable healthcare resources.

To the author's knowledge, there are no known studies which examined the effect of statins and skin infections specifically. Adopting a translational (basic to applied, or "bench-to-bedside") research framework, the overall objectives of this research (in green boxes; Figure 1-1) involved determining if basic research (whether statins exerted *in vitro* antibacterial effects against bacterial skin pathogens) aligned with the

results of applied research (whether statins demonstrated beneficial effects in the ambulatory and hospital care of patients with SSTIs).

Specific research projects (in blue boxes; Figure 1-1) were thus undertaken to accumulate literature evidence on statins' *in vitro* antibacterial effects (Chapter Two), laboratory evidence to evaluate statins' antibacterial effects on skin pathogens (Chapter Three), clinical evidence from the community setting (Chapter Four), and clinical evidence from the hospital setting (Chapter Five). The accumulated evidence was analysed and discussed collectively (Chapter Six), then conclusions were derived as to whether repurposing statins as novel adjuvants/treatments for SSTIs was feasible, along with recommendations for further research (Chapter Seven). If repurposing was found to be viable, statins would potentially serve as AMR breakers and save substantial healthcare resources which could be diverted to other medical conditions.

1.2 Background

1.2.1 Pathogenesis of SSTIs

The human skin confers an initial innate defence against pathogenic microorganisms by functioning as a mechanical barrier due to the tight junctions between epithelial cells, secreting acidic fluids and fatty acids which deter microbial growth, and interacting with its normal flora to impede colonisation by other microbes.[8, 9] When the epidermal protective layer is compromised, the skin initiates cutaneous innate and adaptive immune defences.[10]

SSTIs ensue when inflammatory lesions, microabrasions, or traumatic insults permit microorganisms to infiltrate the protective barrier, and these pathogens adhere to deeper tissue layers of the host, proliferate by escaping the host's immune defence, and produce toxins which overstimulate the human immune system, triggering massive inflammatory responses.[8, 11]

Although SSTIs may be caused by bacteria, fungi, viruses, or parasites, this research focused on bacterial SSTIs due to their predominance over the other types of pathogen-induced SSTIs.[8] In addition, the skin microbiota is composed of mainly

bacteria such as *Staphylococcus aureus*,[12] and colonisation of the skin with *S. aureus* increases the risk of invasive infections.[10]

1.2.2 Bacterial pathogens involved in SSTIs

Being in constant contact with environmental microorganisms, the human skin serves as a primary defence barrier against potential bacterial pathogens. Gram-positive bacteria such as *S. aureus*, *Streptococcus pyogenes*, *Staphylococcus epidermidis*, *Corynebacterium* species and *Propionibacterium* species usually colonise skin surfaces both above and below the waist, while Gram-negative enteric bacteria such as *Enterobacteriaceae* species and *Enterococcus* species usually colonise skin below the waist, likely because of proximity to the anorectal area (faecal veneer).[8, 13]

S. aureus has been identified as the most common bacterial pathogen causing SSTIs,[13] responsible for SSTIs acquired in both the community and hospital.[14] *S. pyogenes* has been implicated for many community-associated SSTIs, while *Pseudomonas aeruginosa*, *Enterococcus* species, *Escherichia coli*, and coagulase negative *S. aureus* are of concern in hospital-associated SSTIs.[14, 15] *Klebsiella pneumoniae* is an opportunistic pathogen of concern that is responsible for community and hospital-acquired infections. Severe skin infections in immunocompromised and diabetic patients have been increasingly associated with *E. coli* and *Serratia marcescens*,[16, 17] whilst patients immunocompromised due to alcohol-induced cirrhosis have an increased susceptibility to developing *Acinetobacter baumannii* associated SSTIs.[18]

1.2.3 Classification of SSTIs

Recommendations have been made to organise SSTIs according to specific variables, such as anatomical location, causative agent, clinical presentation (primary or secondary infection), extent of condition (localised or disseminated), progression rate (acute or chronic condition), and severity (presence of comorbidities).[13] Depending on the depth of infection, SSTIs may be further classified as uncomplicated superficial infections (limited to the epidermis and/or dermis, such as impetigo, folliculitis, or carbuncles), or complicated deep infections (involving the deep dermis, subcutaneous tissue, fascia, and/or muscle, such as cellulitis, myositis, or necrotising infections).[13]

However, a general consensus on the preferred classification for SSTIs has not been reached,[13] probably because of the dynamic and complex nature of SSTIs. Uncomplicated superficial infections may deteriorate to complicated life-threatening infections (especially in immunosuppressed patients), or superficial infections at certain anatomical locations may need to be treated as complicated SSTIs, such as rectal abscesses, which carry a high risk of anaerobic and Gram negative infections.[13]

Although the United States Food and Drug Administration (FDA) introduced the term "acute bacterial skin and skin structure infections" (ABSSSIs) to include cellulitis, wound infections, and cutaneous abscesses with a lesion size of at least 75cm^2, the ABSSSI classification was not utilised throughout the country as the Infectious Diseases Society of America categorised SSTIs by the presence of purulence and disease severity instead.[19] Moreover, the ABSSSI classification excluded chronic polymicrobial infections such as diabetic foot infections, and milder SSTIs such as impetigo.[20] To avoid multiple and ambiguous nomenclatures, this thesis used the term "SSTIs", which may be further specified as superficial, deep, uncomplicated, or complicated where necessary.

1.2.4 Treatment of SSTIs

Depending on the type and severity of infection, early surgical intervention may be required to clean the wound.[9] Upon establishing where and how the infection originated, empirical antibiotics with a spectrum effective against the most likely pathogen(s) are initiated, and changed if required according to culture and sensitivity tests.[13] Empirical treatment for SSTIs should always be effective against *Staphylococcus* species and *Streptococcus* species (normal skin flora), but treatment for SSTIs below the waist should be also effective against *E. coli*, *Enterococcus* species, and other coliforms (faecal veneer).[8]

A short course of topical and/or oral antibiotics may be sufficient for superficial uncomplicated SSTIs, but oral and/or parenteral antibiotics for a longer duration (depending on causative pathogen, infection severity and patient response) are usually required for deep complicated SSTIs.[13] There are currently no guidelines on how long antibiotics should be used to treat bacterial SSTIs, hence the duration of

therapy is usually based on the severity of SSTIs and clinical response of the patient during physician follow-up sessions, with the average treatment ranging from 7 to 14 days.[8, 13]

Extracellular streptococcal toxins contribute to tissue damage, shock, and organ failure, hence attenuation of toxins may improve patient outcome.[21] The role of intravenous immunoglobulin has not been established,[21] but it has been used together with surgical debridement to manage streptococcal toxic shock syndrome because the immunoglobulin may theoretically bind to the exotoxin, neutralise streptococcal superantigens, and aid the host's immunity in clearing *S. pyogenes*.[13]

In addition, wound healing measures have been undertaken to significantly improve patient recovery.[9] Hyperbaric oxygen might improve wound healing,[13] but its effectiveness as a direct treatment for SSTIs is controversial.[22]

1.3 Research problems and question

1.3.1 SSTIs diminish healthcare resources

SSTIs are one of the most frequent forms of infections across different age groups and consume considerable resources in both outpatient and inpatient care.[14] The number of visits to the outpatient clinics and hospital emergency department for SSTI treatment could only be estimated as over 14 million per annum in the United States of America,[11] as it is difficult to accurately determine the incidence of SSTIs due to their brief and diverse presentations.[13] However, it has been reported that in the United States, 14.5 million cases of cellulitis annually resulted in ambulatory costs of $3.7 billion,[23] while the total costs of hospitalisation due to SSTIs caused by *S. aureus* were approximately $4.5 billion for the year 2009, with the incidence of such hospitalisations expected to rise.[24]

The high prevalence of SSTIs is likely due to the myriad of environmental and patient-related risk factors. Environmental risk factors include lifestyle or occupational activities involving close contact with SSTI patients, increased risk of skin colonisation by pathogenic microorganisms, and/or increased risk of trauma to the skin.[13] Patient-related risk factors include diverse susceptible populations such as paediatrics or geriatrics, patients who are alcoholics or obese, patients with

cardiovascular diseases, chronic liver and kidney diseases, diabetes mellitus, compromised immunity, and/or who have peripheral vascular insufficiency.[13] Such patient-related risk factors could influence treatment responses and may be associated with poorer prognosis, accelerated deterioration of disease, more resistant pathogens, and delayed healing.[8, 13]

Diabetic leg infections, nosocomial infections, head and hand infections, and severe SSTIs have been correlated with escalated morbidity and mortality rates, and increased financial burden as a result of greater need for surgery, longer antibiotic treatment, and prolonged inpatient stay.[14, 22] Even upon recovery, patients with diabetes or previous SSTI episodes are at risk of future *S. aureus*-related SSTIs, which predisposes to recurrent SSTIs,[11] resulting in a vicious cycle which further depletes healthcare resources and increase antibiotic usage.

1.3.2 SSTIs associated with AMR

In the ambulatory setting, uncomplicated SSTIs are one of the most common causes of antibiotic prescribing, potentially resulting in excessive and often avoidable antibiotic exposure.[25] Without guidelines for the duration of antibiotic treatment for SSTIs,[13] inappropriate prescribing will likely contribute to the risk of AMR.[26]

The increased use of antibiotics or protracted hospital admissions predispose patients to infections by resistant microorganisms such as methicillin-resistant *S. aureus* (MRSA), methicillin-resistant coagulase negative *S. aureus* (MRCoNS), *Enterobacteriaceae* species (including *E. coli*), *Enterococcus* species, or *P. aeruginosa*.[8, 15]

Given the diverse species of bacteria involved in SSTIs and the various circumstances under which SSTIs may contribute to AMR, the incidence and resistance rates of common pathogens have been reported in various studies on SSTIs as: *S. aureus* (incidence = 23% to 61%, resistance = 25 to 74%); *S. pyogenes* (incidence = 4% to 32%, resistance = 1% to 3%); *P. aeruginosa* (incidence = 14% to 62%, resistance = 7% to 48%); *E. coli* (incidence = 3% to 15%, resistance = up to 28%); and *K. pneumoniae* (incidence = 6% to 10%, resistance = up to 6%).[27]

The pathogens responsible for SSTIs may also be associated with other infectious diseases, thus resistance caused within the SSTI context would extrapolate to AMR in general. *S. aureus* may also cause life-threatening conditions such as bacteraemia, pneumonia, and sepsis.[10] Emergence of resistant *S. aureus* as MRSA complicates treatment and impedes patient recovery due to the pathogen's growing resistance to multiple antibiotics.[10] Its recent prevalence as community-associated MRSA in many parts of the world is perturbing, contributing substantially to the rising incidence rates of SSTIs, increased virulence via toxins such as Panton-Valentine leucocidin and alpha-haemolysin (α-toxin), together with its ability to infect usually healthy people.[10, 28]

The group of *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species have been commonly referred to as "ESKAPE" microorganisms, due to their growing ability to "escape" the effects of many antibacterial agents as multidrug resistant bacteria (non-susceptible to at least one agent in three or more antimicrobial categories).[29, 30] These highly resistant ESKAPE pathogens have been responsible for many life-threatening nosocomial infections around the world.[29] In particular, systemic infections due to microorganisms producing extended-spectrum β-lactamases such as *E. coli* and *K. pneumoniae* have been reported to be independent risk factors for delayed administration of effective antibiotics, extended hospital stay, increased inpatient care costs, and mortality.[29]

The threat of AMR has been deemed similar to that of global warming and terrorism.[5] With the growing trend of resistant pathogens in both the community and hospital setting, there are fewer effective treatment options available, hence the risk of increased morbidity and mortality. The situation is more critical when coupled with a severe deficiency of effective new antimicrobials.

1.3.3 Urgent need for novel treatments

Despite the dire demand for new antibiotics, research and development of such novel agents has not been on the priority list of pharmaceutical companies due to strict drug approval regulations, meagre investment returns, and technical difficulties.[31]

1.3.3.1 Obstacles to development of new antimicrobials

After a public scare of telithromycin which caused a very rare but potentially fatal adverse event (hepatotoxicity) in 2006, FDA regulations on clinical trials tightened considerably, posing stringent regulations for new antimicrobial drug approvals.[32] For infectious diseases, withholding treatment in the inactive drug group of placebo-controlled clinical trials is unethical, hence trails to prove non-inferiority to existing antibiotics had to be conducted, requiring large sample sizes to achieve satisfactory statistical significance, accompanied by substantial expenses.[31]

Potential returns from investments are limited as antibiotics are usually used only for short durations, compared to drugs used long term to treat chronic conditions such as hypertension.[33] Besides the costly labour and time intensive pre-clinical and clinical trials involving large sample sizes, other financial considerations which may substantially reduce profits include most antibiotics being no longer under patent and thus sold as cheaper generics, restricted antibiotic prescribing due to antimicrobial stewardship in hospitals, and economic crises curtailing antibiotic development resources via the reduction of academic research funding and mergers of pharmaceutical companies.[31, 33] Closure of departments in universities and pharmaceutical companies with specialised antibiotic research and development expertise resulted in the gradual loss of relevant skills and knowledge for over more than 30 years,[34] further impeding the potential of new antibiotic development.

The path of new antibiotic discovery has also been fraught with scientific challenges. New classes of antibiotics would be expected to be effective against a broad spectrum of bacteria, especially against the hazardous multidrug resistant pathogens such as the ESKAPE pathogens. Hence, new agents which are able to overcome the resistance mechanisms of current pathogens need to be identified. Genomics-based drug discovery involves determining the genetic codes of critical proteins essential for bacterial survival but non-essential to humans, then referencing these codes against compound libraries to find potential molecules which may bind to these critical bacterial proteins.[35] Although this method is theoretically viable and initially received much financial support from pharmaceutical companies, it lost traction when no viable antibiotics were produced via this method after 20 years.[35]

1.3.3.2 Efforts to develop novel treatments

Realising their stringent regulations on clinical trials contributed in part to the AMR crisis, the FDA reviewed and established a policy reform that focused on benefiting patients with infections caused by extensively drug resistant (susceptible to only one or two antimicrobial categories) or pan-drug resistant (non-susceptible to all agents in all antimicrobial categories) bacteria.[30, 32] However, other measures had to be undertaken as the reform did not provide sufficient impetus for pharmaceutical companies to revive research and development of new antibiotics.[32]

Current research in this area is now centred on novel classes or mechanisms of antimicrobial action such as peptides, bacteriophages, and attenuation of bacterial virulence via interference with signalling molecules which regulate bacterial gene expression according to bacterial population (i.e. quorum sensing).[3] However, these research fields have also encountered their own challenges, namely: (i) antimicrobial peptides being costly, toxic to human cells, and susceptible to proteolysis; (ii) bacteriophages being targeted by the immune system; and (iii) bacteria developing resistance against bacteriophages and quorum sensing inhibitors.[3]

One of the more promising developments has involved the repurposing of existing non-antibiotic drugs for infectious disease treatment, with drugs such as statins (used for treating high cholesterol), terfenadine (allergies), and zafirlukast (asthma) demonstrating *in vitro* efficacy at attenuating growth and/or virulence factors of bacteria.[3] By repurposing existing non-antibiotic drugs as novel antimicrobials or virulence inhibitors, significant savings in time, labour, and financial resources can be achieved since such drugs already have pharmacokinetic, pharmacodynamics, and post-marketing safety data established through clinical trials and usage.[4]

It has been suggested that AMR may be reduced or "broken" by repurposing certain non-antibiotic drugs to augment the antimicrobial effects of failing antibiotics, as proven by the co-administration of β-lactamase inhibitors with β-lactam antibiotics, such as clavulanic acid with amoxicillin respectively.[5] Such non-antibiotic drugs may act as AMR breakers by possessing direct antibacterial activity, synergise with

antibiotics to overcome resistance mechanisms, and/or be able to stimulate the human immune system.[5]

1.3.4 Repurposing statins for SSTIs?

Statins, the common name for 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitors, are taken daily by almost 200 million people worldwide for the primary and secondary prevention of cardiovascular disease.[7] The use of statins for cardioprotection and their adverse effects have been reviewed and established.[36] By competitively binding to HMG-CoA reductase in a dose-dependent manner, statins inhibit the rate limiting step of the mevalonate pathway, thus diminishing cholesterol production.[37]

In the process however, important downstream isoprenoid intermediates such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate (FPP) are also reduced, hence decreasing cell signalling proteins (e.g. Ras, Rac, and Rho) and causing multiple cholesterol-independent (pleiotropic) effects which are cardioprotective (e.g. antithrombotic, antioxidant, antiplatelet, and endothelial protection) and immunomodulatory (e.g. anti-inflammatory, neutrophil extracellular trap [NET] production, and improved wound healing).[38-41]

Of particular interest, statins have been reported to possess the three aforementioned properties of AMR breakers: direct antibacterial activity against methicillin-sensitive *S. aureus* (MSSA) and MRSA,[42] synergism with topical antimicrobials (mupirocin, fusidic acid, retapamulin, and daptomycin) against multidrug-resistant strains of *S. aureus*,[43] and the ability to stimulate the human immune system by enhancing production of NETs.[40] Together with their reported antibacterial activity against *E. coli*, *Enterococcus*, and *Streptococcus* species,[44] anti-inflammatory effects which modulate sepsis,[45] ability to augment wound healing,[41] and suppress toxins such as Panton-Valentine leucocidin and alpha-haemolysin,[46] statins should theoretically be potential AMR breakers and effective therapeutic agents for SSTIs.

As such, the following research projects (Chapters Two to Five) were conducted to provide *in vitro* and *in vivo* evidence in a translational research framework to address the research question of whether statins may potentially be repurposed as viable

novel adjuvants/treatments for bacterial SSTIs, which could help curb AMR and save significant healthcare resources.

CHAPTER TWO

2. Literature Review on Statins' Antibacterial Effects

2.1 Preamble

A comprehensive literature search to review currently published literature on statins' direct antibacterial activity was conducted by the primary investigator in accordance with the evidence-based Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standards (Appendix 1). The work was published as a narrative literature review in the open access and peer-reviewed journal *PeerJ*,[47] under the terms of Creative Commons Attribution 4.0 International Public License, which permits sharing and adaptation of the work if appropriate credit is given, link to the license is provided, and any changes indicated.[48]

Relevant parts of the review paper have been edited and presented in this chapter from Section 2.2 onwards to facilitate flow of the thesis. All spellings have been changed from American to British spelling, labels for references and figures have been amended to align with the format for this thesis, and the two detailed tables from the published review are presented as Appendices 2 and 3 (with corresponding thesis reference numbers), whilst summarised tables (Tables 2-1 and 2-2) have been added in the Results (Section 2.3) for quick reference. To promote transition between thesis chapters, the abstract and introduction sections of the original paper have been abridged and adapted in this preamble, the original section on "Postulated mechanism derived from structure-activity relationship analysis" and corresponding original Figure 3 have been omitted in this chapter because the mechanism has been analysed later in Chapter Three. The original conclusion has been revised to facilitate flow to the following chapter.

All authors had no competing interests to declare. The primary investigator performed the literature and reference searches, collected the data, prepared the figures and tables, wrote the manuscript, and contributed significantly to the design, analysis, and interpretation of findings as lead author in the peer-reviewed publication. Permission was obtained from all co-authors to include the contents of the published paper for this thesis (Appendix 4).

2.1.1 Objectives

A detailed review of current literature was performed to evaluate the effect of statins on AMR and identify if there was sufficient evidence to support statins as novel antimicrobial agents.

Statins may possess traits which appear to align with properties of AMR breakers, namely direct antibacterial activity, synergism with antibiotics to overcome resistance mechanisms, and/or the ability to stimulate the human immune system.[5] This potential of statins as AMR breakers, which albeit promising, could be limited by AMR acquired via selective pressures due to exposure of susceptible bacteria to varying concentrations of statins in the human body and the environment, ironically culminating in statins contributing as AMR "makers" instead.

Statins' potential roles as AMR breakers, AMR makers, and knowledge gaps were thus reviewed as a statin-bacteria-human-environment continuum. From the MIC data available in literature, the susceptibility of various bacteria to individual statins may be ascertained to reveal the most suitable statin for repurposing as a novel adjuvant antimicrobial.

2.1.2 Potential significance of review

By accumulating *in vitro* minimum inhibitory concentration (MIC) results of statins against various bacterial strains reported, the potential of statins as AMR breakers could be evaluated and knowledge gaps identified. If statins had potential to be repurposed as a novel adjuvant antimicrobial, further research projects involving laboratory work (basic science research) and collection of ambulatory and hospital clinical data (applied research) could be planned to bridge the gaps and address the research question of whether statins could serve as novel antibacterial adjuvants/treatments for SSTIs.

2.2 Methods

2.2.1 Literature search

The keywords "statin" or "statins" were combined with "minimum inhibitory concentration" to identify studies which reported MIC values of statins when tested

against specific bacterial strains. "Minimum inhibitory concentration" was used as a keyword instead of a general term "antibacterial effect" because MIC values allow quantitative comparisons of antibacterial potency between individual statins.[49] Moreover, exposure of susceptible bacteria to antibacterial drug concentrations ranging from within eight to ten times above MIC to several hundred times below MIC may contribute to selective pressures for resistance,[50, 51] a theory which could also be applicable to statins, which exert MICs against bacteria. The search was performed by the primary investigator (HK) in six databases on 7th April 2017, namely the Cumulative Index to Nursing and Allied Health Literature (CINAHL), Cochrane Library, Embase, PubMed, Google Scholar, and Web of Science (Figure 2-1).

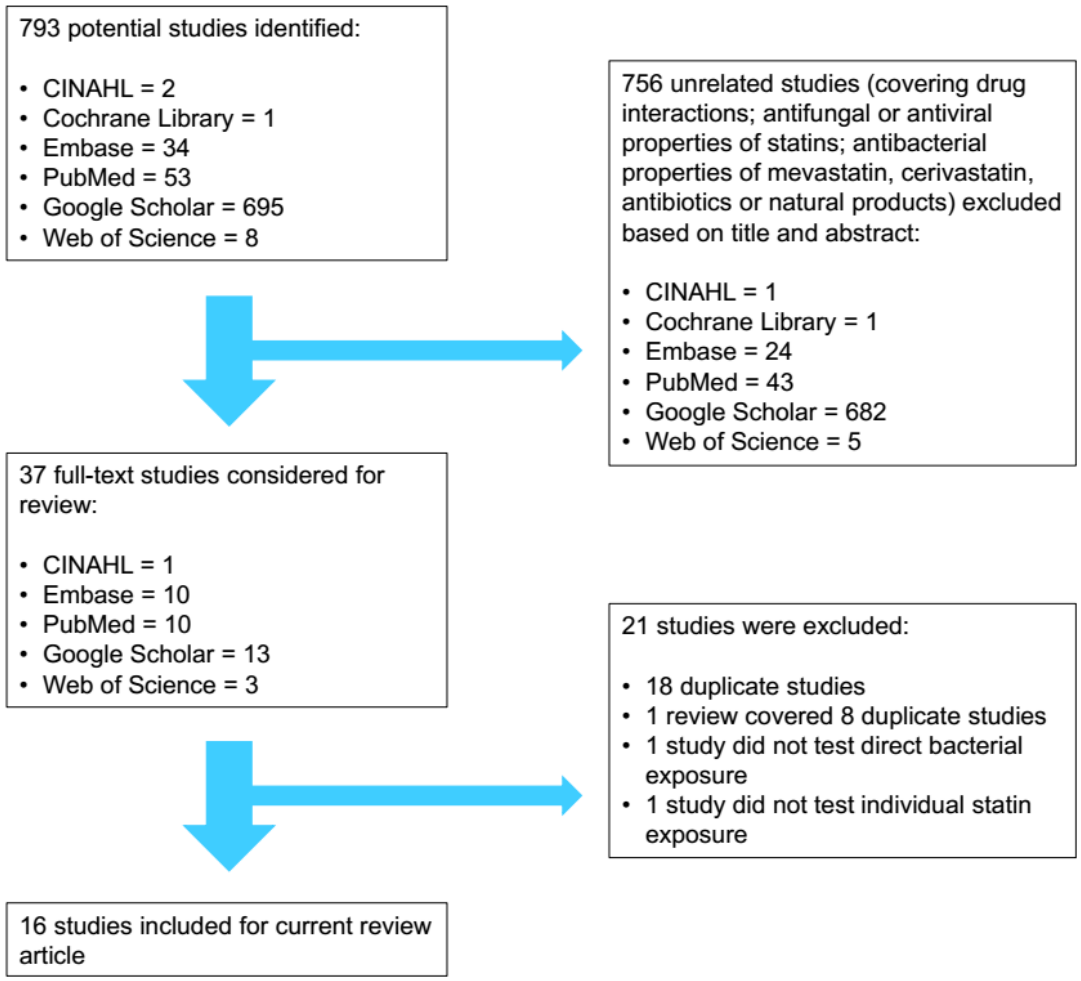

Figure 2-1: Flow chart summarising literature search process performed in six databases on 7th April 2017.

2.2.2 Studies selection

Screening the titles and abstracts of the initial 793 results identified from the keywords, 756 studies were excluded because they covered unrelated topics such as drug interactions; antifungal or antiviral properties of statins; and antibacterial properties of mevastatin, cerivastatin, antibiotics, or natural products. Although antibacterial effects of mevastatin and cerivastatin have been studied,[46] they are not currently used clinically and were therefore omitted in this review.[52] Only antibacterial properties of atorvastatin (ATV), fluvastatin (FLV), lovastatin (LVS), pitavastatin (PTV), pravastatin (PRV), rosuvastatin (RSV), and simvastatin (SMV) were considered relevant for this review as these are currently registered drugs for lowering cholesterol in humans, thus likely to affect the statin-bacteria-human-environment continuum.

Upon reviewing the full text of the remaining 37 studies, 21 studies were further excluded as they contained duplicate information; studied the effects of statins on infected cells instead of direct bacterial exposure; or tested the combined effects of statins and antibiotics without reporting the MIC of statins alone. The resultant 16 pertinent studies consisted of a thesis,[53] a letter with unpublished MIC data,[54] a Turkish study with relevant data in its English abstract,[55] a patent application,[56] a review article with information from a reference in press,[57] and 11 *in vitro* studies.[42-44, 58-65] No new relevant studies were found after scrutinising the references of these 16 studies. The relevance of references was reviewed by all the researchers.

2.2.3 Data extraction

From the 16 selected studies, the MIC values of statins against various Gram-positive and Gram-negative bacteria were detailed in Appendices 2 and 3 respectively, and summarised in Tables 2-1 and 2-2. The dilution methods for Alshammari,[53] Bergman et al.,[58] Quivey,[56] Welsh et al.,[65] and Ting et al.[57] were described in the respective studies. All other studies were tested according to the broth microdilution method stipulated by the Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards (NCCLS). The solvent types and solvent concentrations for water insoluble statins (ATV, LVS, PTV, and SMV) were listed wherever available, because different solvents or solvent concentrations may affect the MIC values.[61]

2.3 Results

2.3.1 Antibacterial activity of statins against Gram-positive bacteria

Statins exhibited antibacterial activity against a wide spectrum of Gram-positive bacteria including oral microbiota (*S. epidermidis*, *Streptococcus anginosus*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *S. pyogenes*, *Streptococcus salivarius*, and *Streptococcus sanguinis*, formerly known as *Streptococcus sanguis*); gut microbiota (*Enterococcus faecalis*, *E. faecium*, *Lactobacillus casei*, and MSSA); drug-resistant bacteria (vancomycin-resistant enterococci [VRE], MRCoNS, MRSA, vancomycin-intermediate *S. aureus* [VISA], and vancomycin-resistant *S. aureus* [VRSA]); and environmental bacteria (*Bacillus anthracis* and *Listeria monocytogenes*) (Table 2-1).

Table 2-1: Summarised range of statins' *in vitro* antibacterial activity against various Gram-positive bacteria reported in literature.

Bacteria type	(Statin name)[Reference(s)]	Lowest MIC (µg/mL) reported	Highest MIC (µg/mL) reported
Bacillus isolates	(ATV)[62]	43.75 ± 17.12	Nil
Bacillus anthracis	(SMV)[43]	16	Nil
Enterococcus faecalis (Vancomycin-resistant)	(ATV)[55, 65]	> 128	250
	(RSV)[44, 65]	100	500 ± 0.00*
	(SMV)[43, 44]	32	291.67 ± 39.53*
Enterococcus faecalis (Vancomycin-sensitive)	(ATV)[44, 60]	83.33 ± 36.08	> 250*
	(PRV)[60]	> 250	Nil
	(RSV)[44, 65]	100	333.33 ± 144.33*
	(SMV)[43, 44]	32	291.67 ± 39.53*
Enterococcus faecium (Vancomycin-resistant)	(ATV)[55]	> 128	Nil
	(SMV)[43, 55]	32	> 128*
Lactobacillus casei	(SMV)[57]	7.8	Nil
Listeria monocytogenes	(SMV)[43]	32	Nil
Staphylococci (Methicillin-resistant coagulase negative, MRCoNS)	(ATV)[55]	> 128	Nil
	(SMV)[55, 56]	64	> 128
Staphylococcus aureus (Methicillin-resistant, MRSA)	(ATV)[43, 62]	37.5 ± 13.98	> 1024*
	(FLV)[42, 43]	> 200	> 1024*
	(LVS)[43]	> 1024	Nil
	(PTV)[43]	> 1024	Nil
	(PRV)[43, 60]	> 250	> 1024*
	(SMV)[44, 60]	31.25	166.67 ± 72.16*
Staphylococcus aureus (Methicillin-sensitive, MSSA)	(ATV)[44, 60, 61]	41.67 ± 18.04	> 250*
	(FLV)[42, 61]	> 200	500
	(LVS)[61]	> 500	Nil
	(PRV)[60, 61]	> 250	> 500
	(RSV)[61, 65]	100	> 500*
	(SMV)[55, 60]	15.65	> 128*
Staphylococcus aureus (Vancomycin-intermediate, VISA)	(SMV)[43]	32	Nil
Staphylococcus aureus (Vancomycin-resistant, VRSA)	(SMV)[43]	32	64
Staphylococcus epidermidis	(ATV)[44]	19.78 ± 4.94	20.83 ± 9.02
	(RSV)[44]	166.67 ± 72.16	233.33 ± 39.52
	(SMV)[44]	26.04 ± 9.02	35.41 ± 4.94
Streptococcus anginosus	(SMV)[57]	7.8	Nil
Streptococcus mutans	(ATV)[53]	100	Nil
	(PRV)[53]	200	Nil
	(RSV)[53]	100	Nil
	(SMV)[53, 56, 57]	15.6	16
Streptococcus pneumoniae	(ATV)[44]	104.17 ± 36.08	229.17 ± 60.38
	(FLV)[58]	> 100	Nil
	(PRV)[58]	> 100	Nil
	(RSV)[44]	333.33 ± 144.33	416.67 ± 0.00
	(SMV)[44, 54]	15	291.67 ± 39.53*
Streptococcus pyogenes	(ATV)[44]	83.33 ± 36.08	133.33 ± 19.76
	(RSV)[44]	166.67 ± 72.16	275.00 ± 72.17
	(SMV)[44]	62.5 ± 0.00	145.83 ± 32.27*
Streptococcus salivarius	(ATV)[53]	100	Nil
	(PRV)[53]	200	Nil
	(RSV)[53]	100	Nil
	(SMV)[53, 57]	7.8	Nil
Streptococcus sanguinis (Streptococcus sanguis)	(ATV)[53]	100	Nil
	(PRV)[53]	200	Nil
	(RSV)[53]	100	Nil
	(SMV)[53, 57]	15.6	Nil

(*) indicates discrepancies in reported MICs by more than two-fold. Further details regarding specific bacterial strains, dilution methods, and solvent/broth used are provided in Appendix 2.

The antibacterial activity of SMV was found to be generally the most potent (lowest MIC) compared to ATV and RSV, especially against *Enterococcus* species ($MIC_{[SMV]} \approx 32$ to 292 μg/mL, $MIC_{[ATV]} \approx 83$ to > 250 μg/mL, $MIC_{[RSV]} \approx 100$ to 500 μg/mL); *Staphylococcus* species ($MIC_{[SMV]} \approx 16$ to 167 μg/mL, $MIC_{[ATV]} \approx 20$ to > 1024 μg/mL, $MIC_{[RSV]} \approx 100$ to > 1024 μg/mL); and *Streptococcus* species ($MIC_{[SMV]} \approx 7.8$ to 292 μg/mL, $MIC_{[ATV]} \approx 83$ to 229 μg/mL, $MIC_{[RSV]} \approx 100$ to 417 μg/mL). FLV exhibited relatively weak antibacterial activity against *Staphylococcus* species ($MIC_{[FLV]}$ between > 200 to > 1024 μg/mL) and *Streptococcus* species ($MIC_{[FLV]} > 100$ μg/mL).

SMV has been the most widely studied, with researchers examining bacteria which were not tested against other statins such as *B. anthracis* ($MIC_{[SMV]} = 16$ μg/mL), *L. casei* ($MIC_{[SMV]} = 7.8$ μg/mL), and *L. monocytogenes* ($MIC_{[SMV]} = 32$ μg/mL). Few studies have been performed on the other statins, but one study did compare the antibacterial effects of all seven registered statins (ATV, FLV, LVS, PTV, PRV, RSV, and SMV) against MRSA and found that only SMV exhibited antibacterial activity ($MIC_{[SMV]} = 32$ μg/mL), while all the other six statins did not (MIC > 1024 μg/mL).[43]

2.3.2 Antibacterial activity of statins against Gram-negative bacteria

As seen in Table 2-2, statins also displayed varying antibacterial activity against a range of Gram-negative bacteria, including oral microbiota (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*); nasopharyngeal microbiota (*Haemophilus influenzae* and *Moraxella catarrhalis*); gut microbiota (*Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, and *Proteus mirabilis*); and environmental bacteria (*A. baumannii, P. aeruginosa*, and *Salmonella Typhimurium*).

Table 2-2: Summarised range of statins' *in vitro* antibacterial activity against various Gram-negative bacteria reported in literature.

Bacteria type	(Statin name)[Reference(s)]	Lowest MIC (μg/mL) reported	Highest MIC (μg/mL) reported
Acinetobacter baumannii	(ATV)[44, 55]	15.62 ± 0.00	> 128*
	(RSV)[44]	300.00 ± 79.05	333.33 ± 144.33
	(SMV)[43, 44]	32.29 ± 6.38	> 256*
Aggregatibacter actinomycetemcomitans	(SMV)[57, 59]	< 1	3.95*
Citrobacter freundii	(ATV)[44]	83.33 ± 36.08	108.33 ± 27.36
	(RSV)[44]	166.67 ± 72.16	333.33 ± 79.06
	(SMV)[44]	52.08 ± 18.04	133.33 ± 39.58
Enterobacter aerogenes	(ATV)[44]	15.62 ± 0.00	19.78 ± 4.94
	(RSV)[44]	104.17 ± 36.08	183.33 ± 0.00
	(SMV)[44]	26.04 ± 9.02	33.33 ± 4.94
Enterobacter cloacae	(ATV)[44]	41.67 ± 18.04	113.54 ± 27.06
	(RSV)[44]	166.67 ± 72.16	316.67 ± 64.55
	(SMV)[44]	62.5 ± 0.00	143.75 ± 36.97*
Escherichia coli	(ATV)[44, 60, 61]	26.04 ± 9.02	> 250*
	(FLV)[61]	500	Nil
	(LVS)[61]	> 500	Nil
	(PRV)[60, 61]	> 250	> 500
	(RSV)[61, 65]	100	> 500*
	(SMV)[44, 61]	52.08 ± 18.04	> 500*
Escherichia coli O157:H7	(SMV)[43]	> 256	Nil
Haemophilus influenzae	(ATV)[44]	83.33 ± 36.08	104.17 ± 36.08
	(FLV)[58]	> 100	Nil
	(PRV)[58]	> 100	Nil
	(RSV)[44]	166.67 ± 72.16	366.67 ± 0.00
	(SMV)[44, 58]	52.08 ± 18.04	> 250*
Klebsiella species (Not specified)	(SMV)[56]	64	Nil
Klebsiella pneumoniae	(ATV)[44, 55]	> 128	216.67 ± 51.03
	(RSV)[44]	258.33 ± 64.55	333.33 ± 144.33
	(SMV)[43, 55]	> 128	> 256
Moraxella catarrhalis	(FLV)[58]	> 100	Nil
	(PRV)[58]	> 100	Nil
	(SMV)[58]	15.6	Nil
Porphyromonas gingivalis	(SMV)[59]	2	Nil
Proteus mirabilis	(ATV)[44]	62.5 ± 0.00	127.08 ± 25.51
	(RSV)[44]	191.67 ± 32.27	250 ± 0.00
	(SMV)[44]	158.33 ± 32.27	166.67 ± 72.16
Pseudomonas aeruginosa	(ATV)[43, 44]	83.33 ± 36.08	> 1024*
	(FLV)[43, 61]	500	> 1024*
	(LVS)[43, 61]	> 500	> 1024
	(PTV)[43]	> 1024	Nil
	(PRV)[43, 60]	> 250	> 1024
	(RSV)[43, 65]	100	> 1024*
	(SMV)[43, 44]	120.83 ± 32.27	> 1024*
Salmonella Typhimurium	(SMV)[43]	> 256	Nil

(*) indicates discrepancies in reported MICs by more than two-fold. Further details regarding specific bacterial strains, dilution methods, and solvent/broth used are provided in Appendix 3.

In general, ATV demonstrated similar or slightly greater antibacterial activity compared to SMV and both were more potent than RSV against *A. baumannii* ($MIC_{[ATV]} \approx 16$ to > 128 μg/mL, $MIC_{[SMV]} \approx 32$ to > 256 μg/mL, $MIC_{[RSV]} \approx 300$ to 333 μg/mL) and *E. coli* ($MIC_{[ATV]} \approx 26$ to > 250 μg/mL, $MIC_{[SMV]} \approx 52$ to > 500

μg/mL, $MIC_{[RSV]}$ ≈ 100 to > 500 μg/mL). FLV exerted relatively weak antibacterial activity against *E. coli* ($MIC_{[FLV]}$ = 500 μg/mL) and *P. aeruginosa* ($MIC_{[FLV]}$ = 500 to > 1024 μg/mL). One study evaluated the antibacterial effects of all seven registered statins against *P. aeruginosa* but did not find any antibacterial activity (MIC > 1024 μg/mL).[43]

2.3.3 Variations in MIC results amongst different studies

An error margin of up to a two-fold difference in MIC is generally acceptable.[66] However, greater differences have been reported in some cases amongst various researchers determining the MICs of statins as indicated by asterisks in Tables 2-1 and 2-2. For example in Table 2-1 when SMV was tested against a specific reference American Type Culture Collection (ATCC) MRSA strain (ATCC 43300) (Appendix 2), the highest $MIC_{[SMV]}$ (≈ 167 μg/mL) and lowest $MIC_{[SMV]}$ (≈ 31 μg/mL) differed by about five-fold.[44, 60] Variations in MIC results of a statin against the same bacterial strain between different studies could be attributed to diversity in materials and methods employed, especially if materials were obtained from different manufacturers. Slight deviations in environmental conditions during manufacture, storage, or transport may affect drug and/or media purity which consequently influences MIC results.

Protocols may not specify every minute detail. General instructions for water insoluble solvents allowed investigators to use various types of solvents and solvent concentrations of their choice, which may result in different MIC results.[61] Most of the studies in Appendices 2 and 3 utilised the CLSI protocol, which recommends an incubation time of 16 to 20 hours for bacteria such as *S. aureus*, but it does not specify if microtiter plates should be subjected to continuous shaking during incubation for broth microdilution methods.[67] A window of 4 hours may result in different MIC results between readings taken at 16 hours compared with 20 hours of incubation. Some researchers may choose to subject the plates to shaking during incubation to facilitate exposure of bacteria to the drug or reduce biofilm formation under static growth conditions. However, continuous shaking during incubation may cause more colonies to grow, affecting MIC results.[68, 69] The CLSI protocol also stipulates that the MIC should be discerned as absence of turbidity with the unaided

eye.[67] This may lead to subjective results, depending on the ability of individuals to detect minute disparities in turbidity.

In view of the multiple factors hampering reproduction of results, it may be more meaningful to compare absolute quantitative results (e.g. MIC) within studies performed by the same researchers, whilst qualitative results or trends (e.g. spectrum of antibacterial efficacy) could be analysed between studies by different researchers.

2.4 Discussion

The positive factors which promote the use of statins as novel adjuvant antibiotics for infections (statins as AMR breakers), the negative factors whereby acquired antibacterial resistance against statins could culminate in AMR (statins as AMR makers), and knowledge gaps are summarised in Figure 2-2 and elaborated as follows.

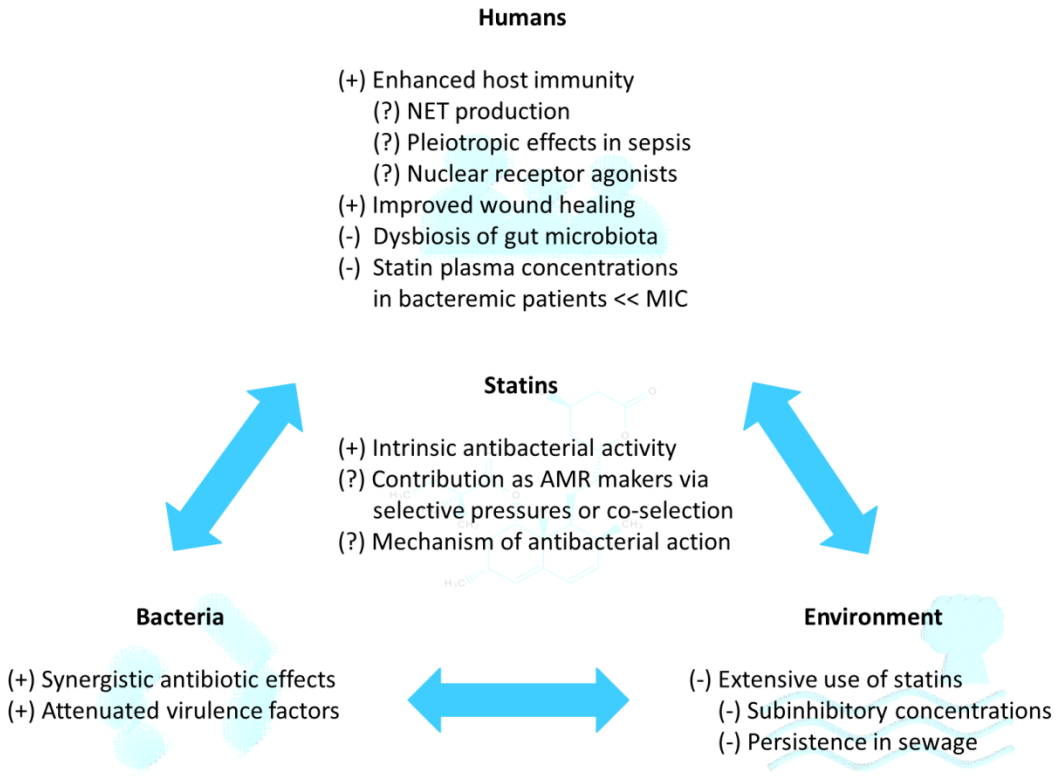

Figure 2-2: Potential of statins as repurposed novel adjuvant antibiotics for infections in the statin-bacteria-human-environment continuum.
(+) refers to factors leading to potentially positive outcomes, whereby statins co-administered with antibiotics may impede AMR (AMR breakers). (-) refers to factors leading to potentially negative outcomes, whereby statin use may favour selective pressures or co-selection for resistance and possibly culminate in AMR (AMR makers). (?) refers to further research required to bridge knowledge gap.

2.4.1 AMR breaker: Intrinsic antibacterial activity

The MIC values in Tables 2-1 and 2-2 provide *in vitro* evidence of individual statins' inherent antibacterial effects against various Gram-positive and Gram-negative bacteria gleaned from literature thus far. SMV has been the most widely studied and demonstrated antibacterial activity against different types of microbiota (oral, gut, and nasopharyngeal) and environmental bacteria (Tables 2-1 and 2-2). SMV also exerted antibacterial effects against Gram-positive drug resistant bacteria such as MRCoNS, MRSA, VISA, VRE, and VRSA (Table 2-1). Therefore, SMV may prove to be an effective antibiotic adjuvant, but *in vivo* studies are required to confirm its clinical antibacterial efficacy.

2.4.2 Knowledge gap: Contribution of statins as AMR makers via selective pressures or co-selection

Despite evidence of statins' intrinsic antibacterial effects, the life span of statins as novel adjuvant antibiotics serving as AMR breakers may be limited due to the widespread use of statins for non-antibiotic purposes (cardiovascular protection). Such extensive usage exposes susceptible bacteria in humans and the environment to varying concentrations of statins, favouring selective pressures for antibacterial resistance. The possible scenarios and repercussions of exposing susceptible bacterial strains to low (up to several hundred times below MIC) and high (within eight to ten times above MIC) statin concentrations are discussed later in this review. Emergence of AMR due to selective pressures are difficult to predict due to variable influences present in humans, animals, and the environment.[70] However, it is certain that the development of AMR occurs naturally in bacteria when exposed to antimicrobials.[71]

Antibiotics, biocides, metals, and non-antibiotic chemicals with antibacterial properties may also induce resistance to multiple antibiotic classes via co-selection.[72, 73] Bacteria may develop multidrug resistance via inheriting genes conferring various resistance mechanisms such as reduced cell permeability to antibiotics, increased efflux of antibiotics, modification of antibiotic targets, or direct inactivation of antibiotics.[71] Co-selection occurs via cross-resistance (selection of a gene conferring multiple resistance mechanisms) or co-resistance (selection of physically linked genes which collectively confer various resistance mechanisms).[72, 73] This is of

particular concern because bacteria may inherit multidrug resistance properties in the absence of selective pressures.[73]

To date, there is evidence that exposure of bacteria to non-antibiotic chemicals with antibacterial properties (chlorite and iodoacetic acid) may induce AMR.[74] Hence, there is a possibility of statins, as non-antibiotic chemicals with antibacterial properties, to similarly contribute as AMR makers, although there is currently little known evidence of such statin associations.

It was found that ATV unlikely contributed to efflux-mediated resistance in multidrug-resistant Gram-negative bacteria.[75] Hence if statins were to induce AMR, it would probably be via other resistance mechanisms. More studies on statins' mechanism of antibacterial resistance, as well as the mechanism of antibacterial activity, are required to determine and thus control the extent of statins' plausible role as AMR makers.

2.4.3 Knowledge gap: Mechanism of statins' antibacterial action

Currently, the mechanism of action for statins' antibacterial effects has yet to be elucidated. The nature of antibacterial activity for SMV against Gram-positive bacteria was found to be bacteriostatic at drug concentrations that equal MIC,[43] but bactericidal at concentrations four times greater than MIC.[60] Suggested mechanisms for statins' antibacterial effects include the pleiotropic effects of statins repressing cell growth,[44] or the hydrophobic nature of SMV disrupting bacterial membrane in a "soap-like" manner,[58] or the reduction of biofilm viability and production.[60] It has also been hypothesised that by lowering host cholesterol levels, statins may reduce the production of a protective membrane-stabilising metabolite in the mevalonate pathway, resulting in bacterial cell toxicity.[76] A postulated mechanism based on the work undertaken for this thesis has been detailed in Chapter Three (Sections 3.4.2 and 3.4.3).

Statins were initially developed with the intention of developing new antibiotics, stemming from the hypothesis that fungi may produce substances which inhibit HMG-CoA reductase, thereby inhibiting the synthesis of isoprenoids essential to microbial life such as cholesterol, thus killing the microorganisms.[77] There are

however, some reasons why statins from fungal origin or the inhibition of HMG-CoA reductase *per se* are unlikely responsible for statins' antibacterial action.

2.4.3.1 Fungal origin unlikely correlates with statins' antibacterial activity

SMV, LVS, and PRV have been classified as Type 1 statins (derived from fungal origins and have similar chemical structures) while ATV, FLV, PTV, and RSV have been classified as Type 2 statins (synthetic compounds with chemical groups which bind more tightly with HMG-CoA reductase).[38] If statins from fungal origins were responsible for the antibacterial activity, then SMV, LVS, and PRV would be expected to exert antimicrobial properties, but not ATV, FLV, PTV, or RSV. Although SMV, LVS, and PRV have similar chemical structures (shown later in Chapter Three, Figure 3-7), SMV exhibited antibacterial properties against *S. aureus* but LVS and PRV do not, despite all three being of fungal origin.[43] Moreover, ATV and RSV are synthetic compounds and not of fungal origin, but both exhibited some antibacterial activity.[44] As such, statins' fungal origin unlikely correlates with their antibacterial activity.

2.4.3.2 Inhibition of human or bacterial HMG-CoA reductase unlikely correlates with statins' antibacterial activity

When administered in humans, all statins competitively bind to the HMG-CoA reductase enzyme in a dose-dependent manner and inhibit the rate limiting step of the mevalonate pathway, thus lowering cholesterol synthesis.[37] If the inhibition of human or bacterial HMG-CoA reductase enzyme contributed towards statins' antibacterial activity, then stronger inhibition of the human enzyme (resulting in higher cholesterol-lowering potency in humans) or stronger binding and inhibition of the bacterial enzyme (theoretically resulting in death due to diminished sterols essential for survival) would correspond with greater antibacterial activity.

However, not all statins exhibit antibacterial activity (Appendices 2 and 3), contradicting the hypothesis that inhibition of human HMG-CoA reductase contributes to antibacterial activity. The presence of the dihydroxy acid moiety is required to competitively inhibit the catalytic function of HMG-CoA reductase and reduce cholesterol synthesis.[78] Statins with lactone groups (SMV and LVS) are prodrugs which must be metabolised to the active dihydroxy acid moiety before they

may inhibit HMG-CoA reductase.[78] Yet SMV, being unable to directly inhibit HMG-CoA reductase, exhibits antibacterial activity against MRSA whilst PRV and PTV, being direct HMG-CoA reductase inhibitors, do not exhibit antibacterial activity.[43]

In addition, the degree of HMG-CoA reductase inhibition corresponds directly with the cholesterol-lowering capabilities of statins,[79] but it does not seem commensurate with antibacterial potency. The cholesterol-lowering potency of statins has been established in the following order: PTV (most potent) > RSV > ATV > SMV > PRV > LVS > FLV (least potent).[80] RSV is a more potent cholesterol-lowering drug compared to SMV, but SMV demonstrated greater antibacterial activity (Tables 2-1 and 2-2), indicating that antibacterial activity may not correlate with the inhibition of human HMG-CoA reductase.

Humans and some Gram-positive bacteria such as *S. aureus* synthesise essential isoprenoids similarly via the mevalonate pathway,[81] depending on the HMG-CoA reductase enzyme as a catalyst in the rate determining step. However, humans and bacteria have different overall HMG-CoA reductase structures.[82] When administered in humans, statins preferentially bind to human HMG-CoA reductase (Class I) instead of bacterial HMG-CoA reductase (Class II) because the affinity of statins is about 10,000 times stronger for human HMG-CoA reductase.[82] This preferential binding of the human enzyme mainly spares inhibition of the bacterial enzyme, permitting the synthesis of essential bacterial sterol synthesis to continue via the mevalonate pathway. Hence, statins are not likely to exert antibacterial effects via inhibition of bacterial HMG-CoA reductase.

Furthermore, many types of Gram-negative bacteria, for example *E. coli* and *P. aeruginosa*, synthesise isoprenoids via an alternative metabolic pathway (2C-methyl-D-erythritol 4-phosphate [MEP]), which do not require HMG-CoA reductase.[81] If inhibition of bacterial HMG-CoA reductase was responsible for statins' antibacterial activity, then it would be expected that statins should exert no antibacterial effect over this class of bacteria, which do not depend on HMG-CoA reductase or the mevalonate pathway for survival. Yet, certain statins (ATV, RSV, and SMV) exert some antibacterial activity against *E. coli*, *P. aeruginosa*, and various other Gram-

negative bacteria (Table 2-2). This suggests statins' antibacterial activity is likely via a mechanism independent of bacterial HMG-CoA reductase inhibition.

2.4.4 AMR breaker: Synergistic antibiotic effects

The combination of antibiotics with drugs that possess direct antibacterial properties or synergistic activity may impede AMR,[5] especially when local delivery of drugs with different mechanisms of action are utilised.[83] SMV exerted synergistic antibacterial effects against *S. aureus* clinical isolates with the topical antibiotics daptomycin, fusidic acid, mupirocin, and retapamulin.[43] However, no synergism was found when SMV was combined with vancomycin against *S. aureus*;[60] when ATV, FLV, LVS, PRV, and SMV were each combined with amikacin, imipenem, or minocycline against *A. baumannii*;[84] or when ATV and FLV were each combined with ciprofloxacin, cefepime, or piperacillin-tazobactam against *E. coli*, *K. pneumoniae*, and *P. aeruginosa* respectively.[84]

2.4.5 AMR breaker: Attenuated virulence factors

Virulence factors enable bacteria to harm the host (via adhesion, invasion, colonisation, and toxin secretion) or protect bacteria from the host's immune defences (via secretion of immune response inhibitors, formation of capsules, and biofilms).[85] Instead of directly threatening bacterial survival with antibiotics that affect essential bacterial genes, it has been suggested that non-threatening approaches such as disarming bacteria by attenuating virulence factors may help reduce AMR.[86]

Through the inhibition of Rho signalling activities and reduced cholesterol production, statins have been observed to attenuate virulence factors. Some examples include reducing bacteria motility and attachment, suppressing production of toxins (Panton-Valentine leucocidin and alpha-haemolysin), directly reducing bacterial translocation and invasion, or protecting against bacterial invasion indirectly via inhibiting lipid raft formation.[46] Statins may also prevent biofilm formation, limit biofilm production, and reduce cell viability in matured biofilms.[60]

2.4.6 AMR breaker: Enhanced host immunity

Stimulation of the host's defence mechanisms to help resolve infections may potentially break AMR.[5, 86] Statins have been shown to directly improve the host's

immune defence in humans as well as in animal models.[40, 87-90] In humans, ATV and SMV may inhibit pro-inflammatory T cells and induce anti-inflammatory T regulatory cells via a novel method involving the downregulation of microRNA let-7c.[91] Clinical studies revealed that SMV enhanced neutrophil function and improved chronic obstructive pulmonary diseases.[89] In addition, women taking statins were less likely to be hospitalised due to the activation of lung macrophage nitric oxide synthase-3, which increases bacterial killing, clearance, and host survival in pneumonia.[90] In animal models, SMV was found to protect mice against *Leishmania major* via augmented phagosome maturation and increased levels of oxidative hydrogen peroxide.[87]

However, statins may also unpredictably influence host immunity via factors such as NET production (Section 2.4.6.1), pleiotropic effects during sepsis (Section 2.4.6.2), and binding as agonists to nuclear receptors (Section 2.4.6.3) as discussed below. More studies are required in these ambiguous areas to determine the overall effects of statins on host immunity and consequently, whether statins potentially break or contribute to AMR.

2.4.6.1 Knowledge gap: NET production

FLV, LVS, and SMV have been shown to produce NETs, which are complexes of nuclear DNA, histones, antimicrobial peptides, and proteases capable of trapping and killing a wide spectrum of microorganisms.[40] However, there is also conflicting evidence that statins do not affect NET production.[92] Further studies may be required to confirm the effect of statins on NETs, as well as whether the NET complexes are in sufficient concentrations to be antibacterial.[92]

2.4.6.2 Knowledge gap: Pleiotropic effects in sepsis

Statins may potentially benefit sepsis by reducing inflammation via intracellular signalling,[45] lowering catecholamine levels,[93] or reducing Toll-like receptor activation by pathogen associated molecular patterns (PAMPs).[94] Statins also possess antiangiogenic (at high doses) and antioxidant effects,[38] which may prevent the progression of severe sepsis.[95] However, sepsis is a complex condition and there have been conflicting results of statins' effects from meta-analysis studies.[96-99]

During early sepsis, high levels of catecholamines and PAMPs such as lipopolysaccharides and lipoteichoic acids cause an initial pro-inflammatory response.[100, 101] An anti-inflammatory response may be initiated concurrent to the initial inflammation and in some cases, secondary infections may cause a secondary pro-inflammatory response.[101] As sepsis continues, pathogenic bacteria may induce vagal stimulation to decrease catecholamines and suppress the host's immune system.[102] There are also many other pro-inflammatory factors (protein catabolism, cachexia, and persistent inflammation) and anti-inflammatory factors (defects in adaptive immunity) that occur slightly later after the onset of sepsis.[103] These variables make it difficult to appropriately administer statins to reduce inflammation or catecholamine levels because it is uncertain if the host is in an overall state of immunostimulation or immunosuppression at any one point in time during sepsis.

Furthermore, the possibility of using statins in infections is further complicated by the potency of statins, whereby different types and doses of statins resulted in different outcomes.[104] At low doses, statins exhibit proangiogenic effects,[38] which may be detrimental in severe sepsis.[95] Hence varying administration times, different types or doses of statin could have caused the conflicting results in meta-analysis studies.

2.4.6.3 Knowledge gap: Nuclear receptor agonists

Statins may indirectly influence the human immune system by binding as agonists to various nuclear receptors, namely farnesoid X receptors (FXRs), glucocorticoid receptors (GCRs), pregnane X receptors (PXRs), and vitamin D receptors (VDRs).[105, 106] Statins may also indirectly induce peroxisome proliferator-activated receptor gamma (PPARγ) activity.[107] The activation of FXRs and VDRs induce antimicrobial peptide gene expression,[108] whilst activation of GCRs, PXRs, and PPARγ result in anti-inflammatory effects.[107-109]

Although statins may bind as agonists to nuclear receptors, a direct increase in nuclear receptor activity may not be apparent because by inhibiting the mevalonate pathway, statins reduce the production of several nuclear receptor agonists such as cholesterol (precursor of glucocorticoids which are GCR and PXR agonists), bile acids (FXR agonist), and vitamin D (VDR agonist).[37] Moreover, nuclear receptors

may also influence the production of other receptor agonists (e.g. activation of PXR reduces bile acid production),[108] and nuclear receptor agonists are not receptor specific (e.g. bile acids are agonists at both FXRs and VDRs; vitamin D is an agonist at GCRs, PXRs, and VDRs).[106, 110, 111]

Some nuclear receptor agonists which boost the human immune system may ironically influence bacterial morphology directly to cause antibiotic tolerance (e.g. bile acids may activate FXRs and VDRs to stimulate antimicrobial peptide production, but bile acids also induce biofilm changes resulting in antibiotic resistant chronic infections).[108, 112] In view of the numerous variables, of which some are antagonistic, it is difficult to anticipate the net effect of statins on the immune system via nuclear receptor activity.

2.4.7 AMR breaker: Improved wound healing

Uncomplicated skin and wound infections are amongst one of the highest causes for outpatient antibiotic usage.[25] As a result, inappropriate or prolonged antibiotic use may contribute to AMR. Antibacterial agents aiding in wound healing should serve to reduce bacterial infection and improve healing time, thus limiting exposure time to antibiotics. Statins are theoretically ideal for wound healing because they may act as PXR agonists to enhance wound healing in intestinal epithelial cells, inhibit FPP (an activator of GCR which impedes wound healing), reduce inflammation, regulate epithelial homeostasis, promote angiogenesis at low doses, reduce oxidative stress, increase vascular endothelial growth factors, and increase levels of nitric oxide.[41, 113-117] The effects of oral statins (ATV, SMV, LVS, PRV, and RSV) and topical statins (ATV, SMV, and LVS) have been examined and it was concluded that there was sufficient evidence to warrant clinical trials assessing the potential efficacy of statins in postoperative wound healing.[41]

2.4.8 AMR maker: Dysbiosis of gut microbiota

Antimicrobials disrupting the gut microbiota may cause AMR and potentially create a store of AMR genes in the gut microbiota, resulting in recalcitrant infections.[118] Statins have been shown to influence gut microbiota diversity in humans,[119, 120] but the mechanism of dysbiosis of the human gut microbiota has not been elucidated. A recent animal study has shown that statin-induced bile acid alterations resulted in

mouse gut dysbiosis via a PXR-dependent mechanism.[121] This review provides plausible evidence that statins may additionally disrupt the human gut microbiota via a direct antimicrobial effect.

From Tables 2-1 and 2-2, Gram-positive (*E. faecalis, E. faecium, L. casei,* and *S. aureus*) and Gram-negative (*C. freundii, E. aerogenes, E. cloacae, E. coli, K. pneumoniae,* and *P. mirabilis*) gut microbiota were susceptible to various statins, whereby $MIC_{[SMV]} \approx 8$ to > 500 µg/mL,[57, 61] $MIC_{[ATV]} \approx 16$ to > 1024 µg/mL,[43, 44] $MIC_{[RSV]} \approx 100$ to > 1024 µg/mL,[43, 65] and $MIC_{[FLV]}$ = > 200 to > 1024 µg/mL.[42, 43]

The licensed oral daily dose range of statins for cholesterol-lowering purposes are SMV = ATV = 10 mg to 80 mg, FLV = 40 mg to 80 mg, and RSV = 5 mg to 40 mg).[80] The laboratory conditions (35 °C and pH 7.2 to 7.4) at which MIC values were determined are attainable when gut microbiota are exposed to statins along the gastrointestinal tract (37 °C body temperature and pH 7.2 to 7.4 along various parts of the small intestines).[67, 122] Although gut concentrations of orally administered parent statin drugs are reduced via absorption, distribution, and metabolism as they move along the gastrointestinal tract, the reduction in concentrations are limited by enterohepatic circulation, and statins are eventually excreted mainly in the faeces (SMV ≈ 60%, ATV > 98%, FLV ≈ 93%, and RSV ≈ 90%).[123, 124] As such, statin concentrations along the gastrointestinal tract are likely sufficient to kill gut microbiota. Even if gut statin concentrations fall below MIC, prolonged gut microbiota exposure to low antimicrobial drug concentrations in general (up to several hundred times lower than MIC) may still result in selective pressures for resistance,[50] a threat which theoretically includes statins as revealed in this scenario.

2.4.9 AMR maker: Statin plasma concentrations in bacteraemic patients being much lower than MIC

Oral doses of statins may be high enough to exert antimicrobial effects in the gut, but the peak statin plasma concentrations have been found to be much lower (SMV ≈ 0.0209 µg/mL, ATV ≈ 0.01 µg/mL, RSV ≈ 0.037 µg/mL, and FLV ≈ 0.24 µg/mL) due to low bioavailability and high protein binding.[42, 65, 125]Comparing these typical peak statin plasma concentrations with MICs in Tables 2-1 and 2-2, the peak plasma concentrations range from hundred to thousand times lower than the reported MICs,

thus likely precluding statins' use as an effective systemic antimicrobial. Of greater concern however, is the risk of exposing bacteraemic patients to such low systemic antimicrobial concentrations, which may result in selective pressures for resistance,[50] a threat which theoretically includes statins as revealed in this scenario.

2.4.10 AMR maker: Environmental impact due to extensive use of stains

The present usage of statins (ATV, RSV, and SMV) has resulted in residual levels (μg/mL to pg/mL) persisting in sewage for at least a few weeks.[126, 127] Since the exposure of bacteria to antibiotic concentrations several hundred times below MIC (in the range of μg/mL to pg/mL) poses a risk of bacterial resistance,[50] this lingering exposure of bacteria in the sewage system to current statin concentrations may thus contribute to selective pressures for resistance.

2.5 Conclusions

The potential roles of statins as AMR breakers, AMR makers, and knowledge gaps in the statin-bacteria-human-environment continuum have been summarised in Figure 2-2. Literature has shown that SMV, ATV, RSV, and FLV exert varying antibacterial effects on Gram-positive and Gram-negative bacteria (Tables 2-1 and 2-2), especially SMV (against most of the Gram-positive bacteria tested) and ATV (against most of the Gram-negative bacteria tested). However, SMV currently appears to be the best candidate as a novel adjuvant antibiotic because it has been the most widely studied statin and demonstrated direct *in vitro* antibacterial activity against various types of microbiota (oral, gut, and nasopharyngeal), drug-resistant bacteria, and environmental bacteria.

Current evidence better supports statins as AMR breakers by working synergistically with existing topical antibiotics, attenuating virulence factors, boosting human immunity, or aiding in wound healing. However, the paucity of data directly associating statins to AMR should not exclude statins' role as plausible AMR makers. The widespread use of statins for non-antibiotic (cardioprotective) purposes may favour selective pressures or co-selection for resistance via dysbiosis of the human gut microbiota, sublethal plasma concentrations in bacteraemic patients, and persistence in the environment, all of which could culminate in AMR.

Perhaps the most urgent knowledge gap to address is determining the mechanism of statins' antibacterial activity. If the antibacterial mechanism involves disarming bacteria instead of directly threatening bacterial survival, AMR is not likely to develop rapidly,[86] and statins may still play an effective role as AMR breakers. However, if the antibacterial mechanism directly threatens bacterial survival, AMR is likely to develop rapidly. If so, statins' role as AMR breakers will likely be limited, and may paradoxically function as AMR makers instead.

These findings provided sufficient evidence to research deeper into the prospect of statins serving as repurposed novel adjuvants/treatments for SSTIs. As such, three further projects were undertaken, namely: (i) laboratory experiments to determine the antibacterial activity and plausible antibacterial mechanism of action of statins against skin pathogens (Chapter Three). (ii) data mining of outpatient prescriptions utilising sequence symmetry analysis ([SSA], Chapter Four), and (iii) a case-control study of hospitalised patients (Chapter Five); to evaluate the association between statin use and the risk of bacterial SSTIs. Reconciling the outcomes from all three studies would help verify if the *in vitro* effects of statins translated to *in vivo* effects, providing evidence that statins may potentially serve as novel adjuvant topical antibiotics.

CHAPTER THREE

3. Laboratory Evidence (Antibacterial Effects Against Skin Pathogens)

3.1 Preamble

From the earlier literature review in Chapter Two, it was found that most of the published studies evaluated the *in vitro* antibacterial activity (determined by the MIC) of only a limited number of statins. There has been only one known study on the antibacterial effects of PTV,[43] one publication on simvastatin hydroxy acid sodium (SMV-OH acid),[61] whilst there is no known data on other statin metabolites such as lovastatin hydroxy acid sodium (LVS-OH acid), pitavastatin lactone (PTV-lactone), or the effect of statins against *S. marcescens.* The wide MIC discrepancies reported by different researchers shown in Tables 2-1 and 2-2 highlighted the importance of adherence to standardised method protocols for meaningful comparison and evaluation of statins' *in vitro* antimicrobial effects. Further, since the reported MICs ranged from hundred to thousand times higher than typical peak statin plasma concentrations, it is unlikely that statins can serve as a safe, effective systemic antimicrobial. However, it may still be possible for statins to be repurposed as a novel adjuvant topical antimicrobial.

Laboratory experiments were thus planned to expand current literature by examining the direct antibacterial effects of all seven statins currently approved for clinical use (ATV, FLV, LVS, PTV, PRV, RSV, and SMV), along with three selected statin metabolites LVS-OH acid, PTV-lactone, and SMV-OH acid, against the most common bacterial strain causing SSTIs (*S. aureus*), and three other strains which may result in complicated SSTIs (*E. coli*, *P. aeruginosa*, and *S. marcescens*).[128] The broth microdilution antimicrobial susceptibility testing methods as stipulated by the CLSI guidelines were employed because the results obtained from these widely recognised standards could be directly compared with most other literature that utilised the same standard.[67] Specific focus on a suitable solvent for statins was considered and recommended for non-water soluble statins to be repurposed as topical antimicrobials.

Although MRSA has been culpable for a significant percentage of SSTIs,[10] the susceptibility of MRSA to statins were not studied in this research as the author worked as a pharmacist in a general hospital and it would be inexpedient to handle resistant microorganisms and risk infecting patients in the hospital. However, the susceptibility of MSSA to statins was examined in detail. Both *E. coli* and *S. marcescens* have been increasingly associated with severe skin infections in immunocompromised patients such as those with diabetes.[16, 17] Since statins may potentially impair β-cell function and decrease insulin sensitivity,[129] determining the susceptibility of *E. coli* and *S. marcescens* to statins would provide relevant information to aid risk/benefit considerations for clinical use.

This work was published as an original research article in the peer-reviewed journal *European Journal of Clinical Microbiology and Infectious Diseases*,[130] under a Copyright Agreement that this post-peer review, pre-copyedit version of the article may be submitted for thesis examination but cannot be made publicly available until after the Embargo Period (i.e. 12 months after 17th May 2018; Appendix 5). The final authenticated version is available online at <http://dx.doi.org/DOI:10.1007/s10096-018-3227-5>.

Relevant parts of the original research article have been edited and presented in this chapter from Section 3.2 onwards to facilitate flow of the thesis. All spellings have been changed from American to British spelling, and the labels for references and figures have been amended to align with the thesis format. The abstract and introduction sections of the original article have been abridged and adapted in this preamble. The original discussion has been extended and edited due to the word limit of the journal, and the original conclusion has been revised in this thesis to promote transition of reading between chapters.

All authors had no competing interests to declare. The primary investigator performed the literature and reference searches, conducted the experiments and collected the data, prepared the figures and tables, wrote the manuscript, and contributed significantly to the design, analysis, and interpretation of findings as lead author in the peer-reviewed publication. Permission was obtained from all co-authors to include the contents of the published article for this thesis (Appendix 6).

3.1.1 Objectives

It has been advocated that the inhibition of bacterial cell growth and determination of the MIC constitutes the standard of early stage antibiotic discovery.[131] As such, the following experiments were conducted to determine the respective MICs of statins and selected metabolites against selected bacterial pathogens responsible for SSTIs.

In addition to identifying if statins exerted bactericidal or bacteriostatic activity, a structure-activity relationship analysis was also performed by reconciling the chemical structure of statins and the selected metabolites with their respective MICs to postulate a plausible mechanism of antibacterial activity.

Topical antibiotics play a key role in the outpatient treatment of uncomplicated SSTIs because the drug may be directly applied to the infected site(s) at concentrations higher than oral or intravenous administration, resulting in reduced risks of systemic adverse effects, less drug interactions, lower healthcare costs, and increased medication compliance.[27] Since the continuous discovery of new topical antimicrobials may help control AMR,[27] the conditions which promote statins as suitable novel topical agents for SSTIs were also explored.

3.1.2 Potential significance of the research

This work not only supplements the available information on statins' *in vitro* antibacterial effects, but also provides a scaffold for future research through discussions of a postulated mechanism of action based on structure-activity relationship analysis, issues on interactions of statins and other antibiotics used to treat SSTIs, addressing the insolubility of statins, choice of solvent for clinical use of novel topical antimicrobial agents, and the possibility of *S. aureus* exhibiting a paradoxical growth phenomenon when exposed to SMV.

3.2 Methods

Bacterial strains used in this study included *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S. marcescens* (ATCC 21074/E-15). Statin powders of at least 98% purity were procured from various manufacturers,

namely Sequoia Research Products (ATV, PTV, PTV-lactone, and RSV), Tocris Bioscience (FLV, LVS, PRV, and SMV), and Toronto Research Chemicals (LVS-OH acid and SMV-OH acid). Acceptable MIC limits for the bacteria were monitored with piperacillin-tazobactam (Alphapharm) and cefazolin (Sandoz) antibiotics.

The susceptibility of bacteria to statins was performed in sterile 96-well microtiter plates (Nunc, Thermo Scientific) utilising broth microdilution and direct colony suspension methods according to the CLSI guidelines.[67] Sterile Mueller-Hinton agar ([MHA], Oxoid) was used for bacterial cultures and colony counting. Sterile Mueller-Hinton broth ([MHB], Oxoid) was supplemented with sterilised calcium chloride (Ajaz Chemicals) and magnesium chloride (Scharlau Chemie) to obtain sterile cation-adjusted Mueller-Hinton broth (CAMHB).[67] A microtiter plate reader (EnSpire, Perkin Elmer) was used to adjust the initial inoculum to 0.5 McFarland Turbidity Standard and for spectrophotometric analyses.

3.2.1 Solvent for water-insoluble statins

Both dimethyl sulfoxide ([DMSO], Fisher Chemical) and methanol (Sigma-Aldrich) possess antimicrobial effects,[132, 133] which may influence the MIC results.[61] Hence, 50 µL inoculum suspensions of *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. marcescens* were each tested with 50 µL of DMSO 2.5%, DMSO 5%, methanol 2.5%, and methanol 5% respectively. Positive growth control (GC) wells (50 µL inoculum + 50 µL of CAMHB) and sterility control (SC) wells (100 µL of CAMHB) were included in triplicates for each experiment. The plates were incubated without shaking at 35°C for 20 hours, and optical density at wavelength 625 nm (OD625) readings were taken before incubation (0 hour) and at two-hourly intervals. The experiment was repeated on another day to obtain two independent results.

3.2.2 Preparation of statins

Water-soluble statins (FLV, PRV, RSV, LVS-OH acid, and SMV-OH acid) were dissolved in sterile purified water as a stock solution, then diluted with CAMHB to obtain ten different final statin concentrations (256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, and 0.5 µg/mL) for each experiment.[67]

From (Section 3.2.1), methanol generally had less suppressive effects on the growth of all strains used in this study. Hence, water-insoluble statins (ATV, PTV, PTV-lactone, LVS, and SMV) were dissolved in 100% methanol to make up several vials of respective stock solutions, each containing 5120 μg/mL drug in 100% methanol. Each working day's final concentrations (256 μg/mL to 0.5 μg/mL with inoculum) for incubation were prepared from a fresh stock vial, diluted with CAMHB according to the method recommended by CLSI,[67] such that the highest final statin concentration (256 μg/mL) contained 5% methanol, while the lower final statin concentrations (128 μg/mL to 0.5 μg/mL) contained 2.5% methanol or less.[67]

For each dilution step, the more concentrated solution was vortexed immediately before sampling, followed by several times of up and down suction with the micropipette during sampling to obtain uniformed dilutions of the drug as far as possible. However, SMV was not completely dissolved at 256 μg/mL and 128 μg/mL. Hence the vortexing and multiple suction action with the micropipette were essential to ensure reasonably accurate dilution and distribution of undissolved drug. The problem of undissolved SMV was further addressed in Section 3.2.6 below.

3.2.3 Broth microdilution method

Each statin-bacteria experiment consisted of triplicate test wells for each of the ten final statin concentrations (specific statin in 50 μL CAMHB + specific inoculum in 50 μL CAMHB), triplicate positive growth control (GC) wells (50 μL inoculum + 50 μL of CAMHB), and triplicate sterility control (SC) wells (100 μL of CAMHB). An aliquot (10 μL) was sampled from a GC well immediately after inoculation and diluted appropriately for colony counting.[67]

Being incompletely soluble at 256 μg/mL and 128 μg/mL, SMV had much higher OD625 readings than the low baseline of the other wells (64 μg/mL to 0.5 μg/mL, GC, and SC) before incubation. As such, it was ensured that the OD625 readings of the triplicate 256 μg/mL wells were comparable amongst themselves, and the same was done for the triplicate 128 μg/mL wells. This was necessary to be reasonably assured that the dilution steps were as accurate as possible and that the undissolved drug was evenly distributed within each of the high concentrations before incubation.

The experimental microtiter plates and MHA plates for colony counting were incubated at 35°C for 20 hours. Continuous shaking of experimental plates was not performed during incubation as this was not specified in the CLSI guidelines.[67] Moreover, shaking may cause an increase in colony growth.[69] All experiments were repeated on separate days to obtain a total of three independent results.

3.2.4 Unaided visual determination of MIC and test for bacteriostatic or bactericidal effects

The MIC is defined as the lowest antimicrobial drug concentration that completely inhibits microbial growth as detected by the unaided eye.[67] After incubation, experimental plates were examined against a dark background and the lowest statin concentrations with clear wells were noted as the MIC. Each experiment was valid only if all GC wells were turbid (indicating bacterial growth); all SC wells were clear (indicating absence of contamination); and the MHA plates showed average colony counts of between 20 to 80 (x 10^4 colony forming units [CFU]/mL), reflecting the inoculum size prior to incubation.[67]

In order to evaluate whether the antimicrobial effect of statins was bacteriostatic or bactericidal, clear cultures of statin concentrations at MIC and higher were further sampled and plated on sterile MHA plates, then incubated at 35°C for 20 hours. The appearance of abundant colony growth after incubation would indicate bacteriostatic activity, while absence of colony growth would suggest statins are bactericidal at the respective drug concentrations from which they were sampled from.

3.2.5 Spectrophotometric analysis

Supplementary spectrophotometry was performed to determine potential antibacterial activity, which may present with significantly lower turbidity compared to GC, but indiscernible to the unaided eye. Turbidity was reported as percentage OD625, whereby OD625 of GC after incubation at 35°C for 20 hours was taken to be 100% for each experiment. Spectrophotometry was conducted at OD625 because the wavelength of 625 nm was used to determine 0.5 McFarland Turbidity Standard, and exposure to this wavelength does not kill *S. aureus* or *E. coli*.[67, 134]

3.2.6 Determining MIC with incompletely dissolved SMV

Spectrophotometry was also necessary in this study because SMV was visibly incompletely dissolved at 256 μg/mL and 128 μg/mL before incubation, which contributed to baseline OD625 readings before incubation. The relative solvent concentrations were the same as the previous method of statin preparation, with the highest final statin concentration (256 μg/mL) containing 5% methanol, while the lower final statin concentrations (128 μg/mL and 64 μg/mL) containing 2.5% methanol or less. The following three methods may collectively help determine if SMV exerted antibacterial effects at these higher concentrations.

3.2.6.1 Effect of undissolved SMV alone during incubation

Monitoring changes in turbidity of undissolved SMV alone in sterile CAMHB during incubation would indicate if SMV was dissolving (decreasing turbidity), remains undissolved (constant turbidity), or precipitating out (increasing turbidity). A microtiter plate consisting triplicate test wells (50 μL SMV + 50 μL sterile CAMHB) of SMV concentrations 256 μg/mL and 128 μg/mL, and triplicate SC wells, was incubated at 35°C for 20 hours. Readings were taken before incubation and at four-hourly intervals. The experiment was repeated on a separate day to obtain two independent results.

3.2.6.2 Effect of undissolved SMV incubated with inoculum during log phase

When undissolved SMV is incubated with inoculum, decreasing turbidity during active *S. aureus* growth at log phase would indicate SMV possesses antibacterial effects. A microtiter plate consisting triplicate test wells (50 μL SMV + 50 μL inoculum) each of SMV concentrations 256 μg/mL and 128 μg/mL, triplicate GC wells, and triplicate SC wells, was incubated at 35°C for 20 hours. Readings were taken before incubation, during exponential growth phase (after 6 and 8 hours of incubation), and after the CLSI-recommended incubation period (16, 18, and 20 hours of incubation). The experiment was repeated on separate days to obtain three independent results.

3.2.6.3 Comparing colony counts before and after incubation

Compared against average colony counts before incubation, similar or lower counts after incubation would indicate SMV exerted antibacterial effects, whilst

significantly higher counts suggest otherwise. Experiments for SMV were repeated to obtain three independent results, each with the additional step of sampling 10 μL aliquots from SMV at 256 μg/mL, 128 μg/mL, and 64 μg/mL after 20 hours of incubation. The aliquots were diluted and incubated at 35°C for 20 hours, after which average colony counts were determined.[67]

3.2.7 Statistical analysis

Statistical data were analysed with GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, California, United States of America). Data for growth curves of bacteria in varying concentrations of solvent were presented as mean ± standard deviation. OD625 readings in varying drug concentrations were presented as mean ± standard error of the mean. One-way analysis of variance (ANOVA) with Dunnett's post hoc test was performed to test for significant differences between GC and the various drug concentrations, whereby $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****).

3.3 Results

3.3.1 Solvent for water-insoluble statins

Compared to methanol at 2.5% and 5%, DMSO at the same concentrations had greater suppressive effects on the growth of all bacterial strains used in this study (Figure 3-1). Hence methanol (maximum 5%) was chosen as the solvent for water-insoluble statins. Although the OD625 reading of *S. aureus* in 5% methanol after 20 hours of incubation was greater than the control experiment in Figure 3-1a, it was not statistically significant (one-way ANOVA with Dunnett's post hoc test). Thus, any increase in *S. aureus* burden in the presence of 5% methanol (statins with concentrations of 256 μg/mL) was unlikely sufficient to affect the MIC results.

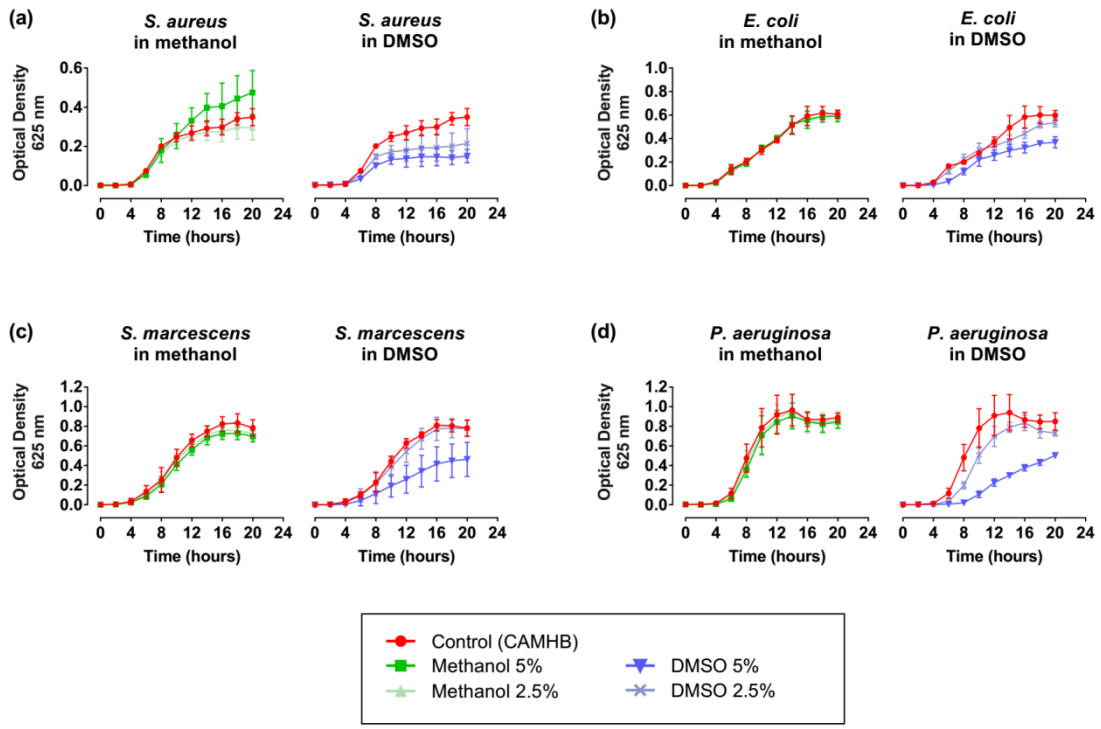

Figure 3-1: Comparing the effects of DMSO and methanol at different concentrations on various bacterial strains.
Effects of solvents were tested on **(a)** *S. aureus*, **(b)** *E. coli,* **(c)** *S. marcescens,* and **(d)** *P. aeruginosa*. Growth of bacteria was monitored as turbidity, measured as OD625. Each panel shows the results of two independent experiments expressed as mean ± standard deviation. [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

3.3.2 Unaided visual determination of MIC and test for bacteriostatic or bactericidal effects

The lowest statin concentrations that completely inhibit bacterial growth (determined by the unaided eye) were presented in Figure 3-2a, whereby *S. aureus* was most susceptible to SMV (MIC = 64 µg/mL), followed by PTV-lactone (MIC = 128 µg/mL), then ATV and FLV ($MIC_{[ATV]} = MIC_{[FLV]}$ = 256 µg/mL). Gram-negative bacteria *E. coli*, *P. aeruginosa*, and *S. marcescens* were not susceptible to any of the statins at concentrations ≤ 256 µg/mL.

(a)

Bacterial strain	Statin (MIC in µg/mL)									
	ATV	FLV	LVS	PTV	PRV	RSV	SMV	LVS-OH acid	PTV-lactone	SMV-OH acid
Gram-positive										
S. aureus	256‡	256‡	> 256	> 256	> 256	> 256	64‡	> 256	128‡	> 256
Gram-negative										
E. coli	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
P. aeruginosa	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
S. marcescens	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256

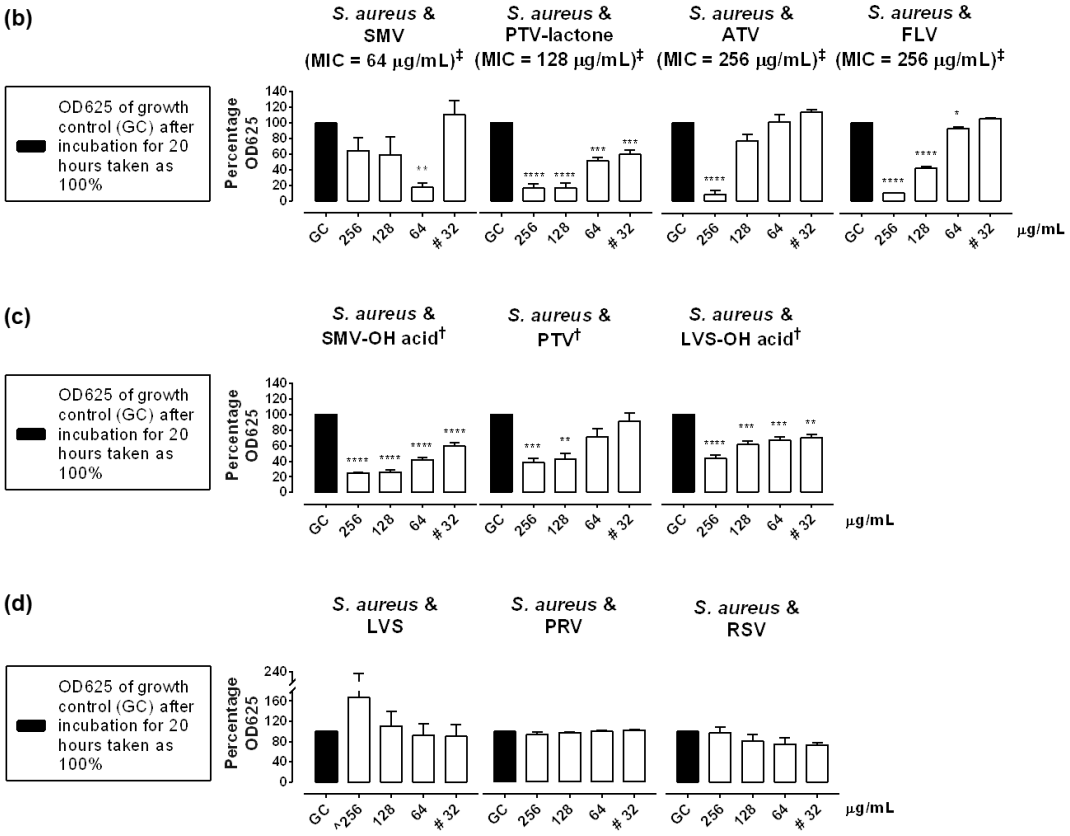

Figure 3-2: Susceptibility of various bacterial strains to specific statins after incubation for 20 hours at 35°C.

(a) Visual determination of MIC. (‡) MIC reported as the lowest statin concentrations (≤ 256 µg/mL) which consistently exhibited no turbidity in three independent experiments as observed by the unaided eye. **(b)** Spectrophotometric results of statins exhibiting MIC against *S. aureus*. Absence of turbidity discerned by the unaided eye corresponded to OD625 < 20% in this study. (#) Statin concentrations lower than 32 µg/mL did not show significantly lower OD625 relative to GC. **(c)** Spectrophotometric results of statins demonstrating potential antibacterial activity against *S. aureus.* (†) Statins with potential antibacterial activity against *S. aureus* (significantly lower OD625 relative to GC detected by spectrophotometry but turbidity indiscernible by the unaided eye). **(d)** Spectrophotometric results of statins demonstrating no antibacterial activity against *S. aureus.* (^) Large OD625 value expressed with a break in the y-axis. For (b), (c), and (d), mean results of three independent experiments were presented, with error bars indicating standard error of the mean. One-way ANOVA with Dunnett's post hoc test was used to compare OD625 differences between GC and the various statin concentrations. Statistically significant results were annotated when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) or $p < 0.0001$ (****). [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

The clear cultures which were sampled from statins with reported MICs, when further plated on sterile MHA plates and incubated to determine bacteriostatic or bactericidal effects, resulted in abundant bacterial growth for all samples (data not shown).

3.3.3 Spectrophotometric analysis

The unaided visual determination of MIC for *S. aureus* (Figure 3-2a) whereby no turbidity was observed, corresponded to turbidity levels of OD625 < 20% (Figure 3-2b). Spectrophotometric analysis detected significantly reduced turbidity at statin concentrations that were lower than the reported MICs, such as for PTV-lactone (32 μg/mL) and FLV (64 μg/mL) (Figure 3-2b). However, since unaided visual observation discerned turbidity at these statin levels, these concentrations could not be reported as MICs in accordance with CLSI guidelines.[67] Similarly, although there was significant reduction in turbidity detected by spectrophotometry for SMV-OH acid, PTV, and LVS-OH acid against *S. aureus* (Figure 3-2c), MIC values could not be reported for these statins. There was no antibacterial activity detected for LVS, PRV, and RSV against *S. aureus* at drug concentrations ≤ 256 μg/mL (Figure 3-2d). In addition, SMV-OH showed statistically significant activity against *E. coli* (Figure 3-3) and *S. marcescens* (Figure 3-4).

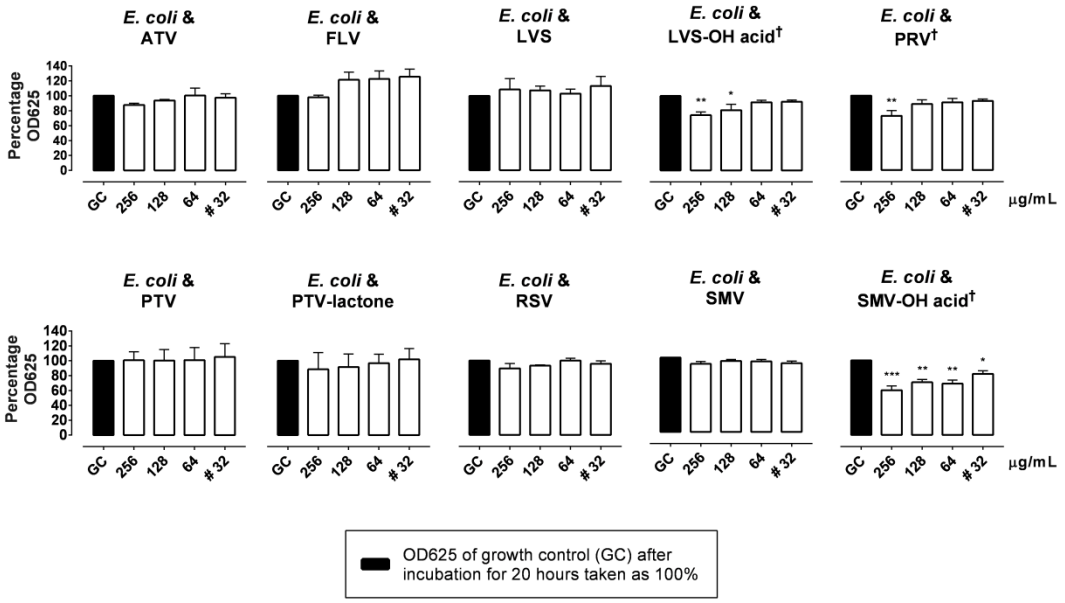

Figure 3-3: Antibacterial activity of statins against *E. coli* after incubation for 20 hours at 35°C as determined by spectrophotometry.
Bacterial growth (turbidity) was expressed as percentage OD625, whereby OD625 of GC (absence of statin) was taken as 100%. (†) Statin with potential antibacterial activity (significantly lower OD625 relative to GC detected by spectrophotometry but turbidity indiscernible by the unaided eye). (#) Statin concentrations lower than 32 µg/mL did not show statistically significant OD625 values relative to GC. Each chart shows the mean OD625 of three independent experiments, with error bars indicating standard error of the mean. One-way ANOVA with Dunnett's post hoc test was used to compare OD625 differences between GC and the various statin concentrations after incubation. Statistically significant results were annotated when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****). [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

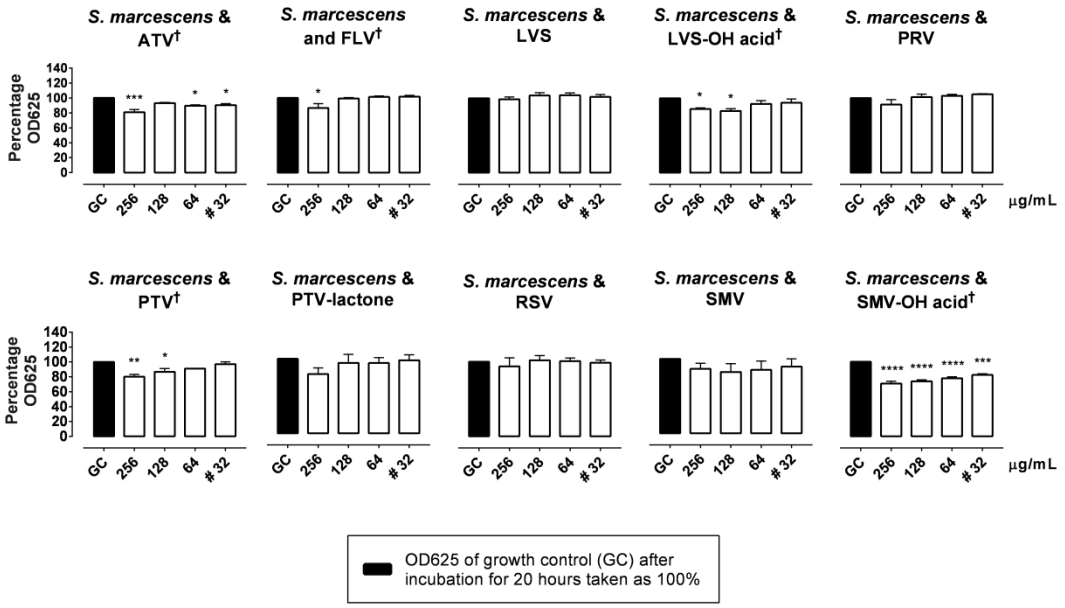

Figure 3-4: Antibacterial activity of statins against *S. marcescens* after incubation for 20 hours at 35°C as determined by spectrophotometry.
Bacterial growth (turbidity) was expressed as percentage OD625, whereby OD625 of GC (absence of statin) was taken as 100%. (†) Statin with potential antibacterial activity (significantly lower OD625 relative to GC detected by spectrophotometry but turbidity indiscernible by the unaided eye). (#) Statin concentrations lower than 32 µg/mL did not show statistically significant OD625 values relative to GC. Each chart shows the mean OD625 of three independent experiments, with error bars indicating standard error of the mean. One-way ANOVA with Dunnett's post hoc test was used to compare OD625 differences between GC and the various statin concentrations after incubation. Statistically significant results were annotated when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****). [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

Finally, no antibacterial activity was detected for any of the statins against *P. aeruginosa* (Figure 3-5).

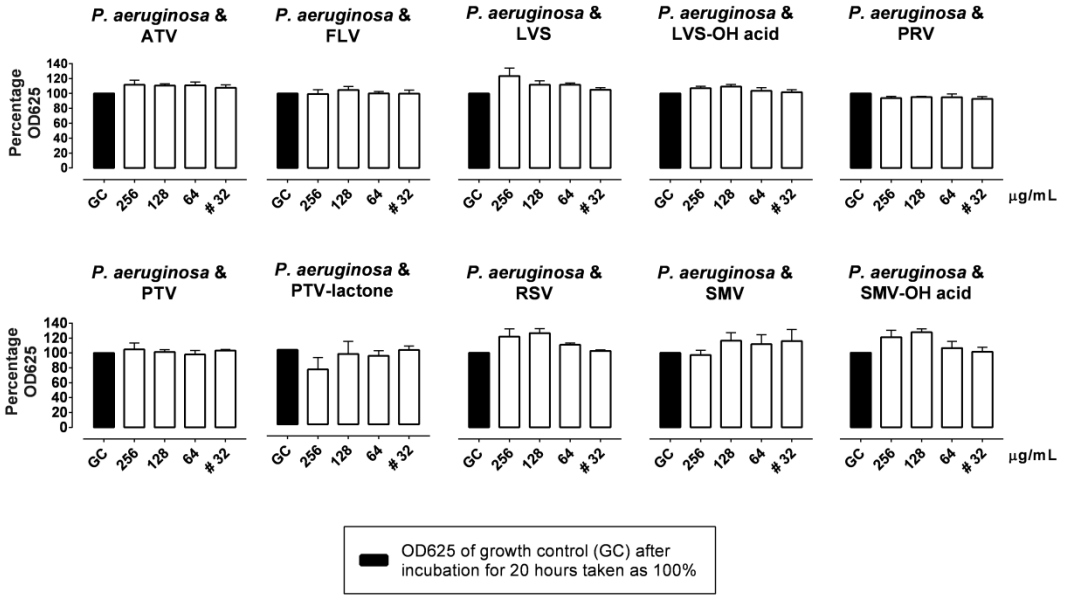

Figure 3-5: Antibacterial activity of statins against *P. aeruginosa* after incubation for 20 hours at 35°C as determined by spectrophotometry. Bacterial growth (turbidity) was expressed as percentage OD625, whereby OD625 of GC (absence of statin) was taken as 100%. (#) Statin concentrations lower than 32 µg/mL did not show statistically significant OD625 values relative to GC. Each chart shows the mean OD625 of three independent experiments, with error bars indicating standard error of the mean. One-way ANOVA with Dunnett's post hoc test was used to compare OD625 differences between GC and the various statin concentrations after incubation. Statistically significant results were annotated when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****). [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

3.3.4 Determining MIC with incompletely dissolved SMV

Incompletely dissolved SMV before incubation was found to dissolve over time (decreasing OD625) during incubation, but after 20 hours of incubation, some undissolved drug remained (residual OD625) for both SMV at 256 µg/mL and 128 µg/mL (Figure 3-6a).

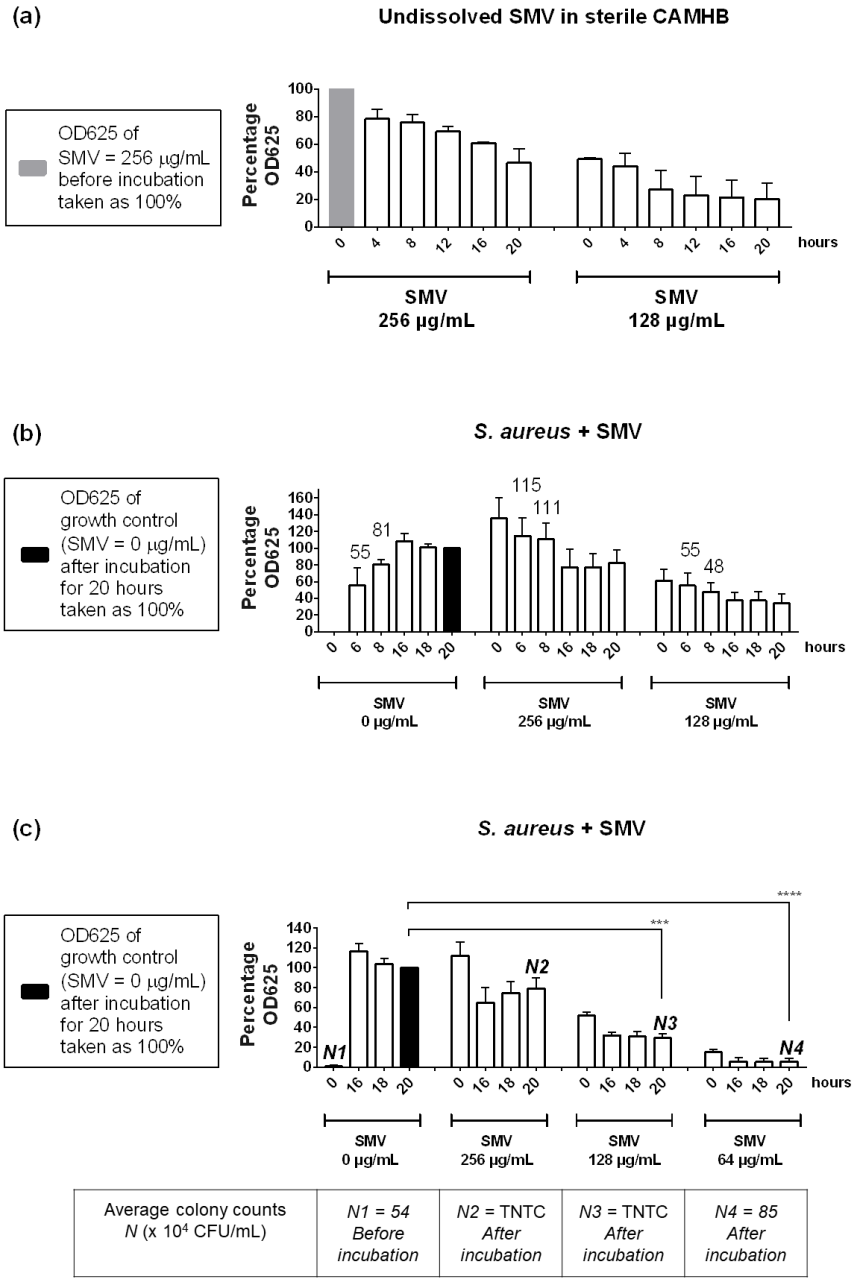

Figure 3-6: Determining MIC with incompletely dissolved SMV.
(a) Effect of undissolved SMV (measured as OD625) at 256 µg/mL (in 5% methanol) and at 128 µg/mL (in 2.5% methanol) in sterile CAMHB during 20 hours of incubation. Two independent experiments were conducted and the results were presented as mean ± standard error of the mean. **(b)** Monitoring the effect of various SMV concentrations (0 µg/mL, 256 µg/mL, and 128 µg/mL) on bacterial growth (measured as OD625) during the estimated log phase of *S. aureus* (between 6 to 8 hours of incubation). The results of three independent experiments were expressed as mean ± standard error of the mean. **(c)** Comparing the average colony counts of the initial inoculum (*N1*, before incubation) against samples after incubation with SMV at 256 µg/mL (*N2*), 128 µg/mL (*N3*), and 64 µg/mL (*N4*). The results of three independent experiments were expressed as mean ± standard error of the mean. One-way ANOVA with Dunnett's post hoc test was used to compare OD625 differences between the positive growth control and the various statin concentrations after 20 hours of incubation. Statistically significant results were annotated when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****). TNTC, too numerous to count. [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

In the absence of SMV, *S. aureus* demonstrated active growth during the log phase between 6 to 8 hours of incubation (Figure 3-1), and as shown by the respective increase in OD625 from 55% to 81% (Figure 3-6b; SMV = 0 μg/mL). However for SMV at 128 μg/mL, OD625 decreased from 55% to 48% between 6 to 8 hours respectively during what would have been the log phase (Figure 3-6b; SMV = 128 μg/mL). The effect of SMV at 256 μg/mL on *S. aureus* growth could not be determined due to excessive cloudiness from the high concentration of undissolved drug, which obscured turbidity changes during what would have been the log phase (Figure 3-6b; SMV = 256 μg/mL; between 6 to 8 hours, OD625 decreased slightly from 115% to 111% respectively).

Upon repeating the experiments to obtain a total of three experiments, the average colony count before incubation (initial inoculum size; *N1* = 54 x 10^4 CFU/mL) was comparable with the average count after incubation for SMV at 64 μg/mL (MIC; *N4* = 85 x 10^4 CFU/mL). However, the average counts after incubation for SMV at 128 μg/mL (*N3*) and 256 μg/mL (*N2*) were both too numerous to count (Figure 3-6c).

3.4 Discussion

3.4.1 Statins suitable as topical antibacterial agents

Against Gram-positive *S. aureus* (ATCC 29213), SMV, PTV-lactone, ATV, and FLV demonstrated bacteriostatic effects, with $MIC_{[SMV]}$ = 64 μg/mL, $MIC_{[PTV\text{-}lactone]}$ = 128 μg/mL, and $MIC_{[ATV]}$ = $MIC_{[FLV]}$ = 256 μg/mL. The MIC results of SMV, ATV, and FLV were similar to other studies,[55, 60, 61, 64] within an acceptable two-fold difference in MIC.[66] At higher concentrations (4 x MIC), SMV has been shown to exert bactericidal effects against *S. aureus*.[60] To our knowledge, there have not been any prior studies on the antimicrobial activity of PTV-lactone. Although SMV-OH did not achieve MIC at concentrations ≤ 256 μg/mL, spectroscopic analysis showed statistically significant activity against *S. aureus*, *E. coli*, and *S. marcescens* (Figures 3-2c, 3-3, and 3-4), which suggests potential antibacterial activity whereby MIC might be achieved at drug concentrations above 256 μg/mL.

For *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853), MIC was not achieved for any of the statins at concentrations up to 256 μg/mL, similar to reports by other

researchers for both strains.[60, 61] However, Welsh et al. demonstrated different results ($MIC_{[ATV]}$ = 250 µg/mL and $MIC_{[RSV]}$ = 100 µg/mL) for both strains, possibly due to the use of a different solvent and concentration (6.25% ethanol) for ATV and different culture medium for the bacterial strains (7% horse blood agar).[65] There have been no other known studies on statins against Gram-negative *S. marcescens* (ATCC 21074/E-15).

The peak plasma concentrations attained for cholesterol-lowering purposes (SMV ≈ 0.0209 µg/mL, PTV-lactone ≈ 0.025 µg/mL),[42, 135] are at least 1,000 times lower than the *in vitro* MIC results reported in our study. This suggests that antibacterial effects are highly unlikely with the oral administration of SMV and PTV-lactone at doses for reducing cholesterol, and attempts to attain such high concentrations via the oral route escalates the risk of systemic toxicity. However, it may be feasible to achieve MIC concentrations by administering SMV and PTV-lactone as topical antibacterials directly onto the site of infection, especially since SMV is possibly effective against *S. aureus* resistant to methicillin or vancomycin as well.[43, 60]

More studies are required to evaluate the safety of using high topical doses of statins, and the likelihood of adverse effects when combining statins with other antibiotics normally used to treat SSTIs, especially fluoroquinolones and macrolides. In particular, ciprofloxacin (a fluoroquinolone) inhibits the liver's cytochrome P450 enzyme system (strong inhibitor of CYP1A2 and weak inhibitor of CYP3A4) to elevate SMV levels, while macrolides may inhibit CYP3A4 and organic anion-transporting polypeptides (uptake transporters) in the liver, and drug efflux pump P-glycoprotein in the intestinal lumen to increase certain statins' concentrations.[136]

The choice of solvents for water-insoluble statins may influence antimicrobial results.[61] Our choice of using methanol as a solvent was based on our finding that methanol exerted less suppressive effects on the bacterial strains tested in this study, compared to DMSO (Figure 3-1). Our results thus supplement other studies which utilised DMSO as a solvent,[60, 64] showing that statins possess inherent antibacterial properties regardless of solvent used. Future clinical research may benefit from using DMSO (up to 10%) as a solvent because it has low toxicity and possesses antibacterial, analgesic, anti-inflammatory, and wound healing properties.[132, 137]

Utilising alcohol as a solvent for clinical use appears unfavourable as it may encourage biofilm formation and antibacterial resistance,[138] or increase the risk of haemolysis in certain staphylococci strains, exacerbating skin infections.[139]

3.4.2 Structure-activity relationship analysis

By comparing the chemical structures of statins with antibacterial activity against those without, the chemical functional groups responsible for antibacterial activity may be identified, providing clues to statins' mechanism of antibacterial activity. The combination of three aspects appear to govern statins' antibacterial activity against *S. aureus*: hydrophobicity of the ring system; a lactone ring or dihydroxy acid moiety; and the presence of a *gem*-dimethyl moiety (two methyl groups on the same carbon atom) or a cyclopropyl ring (Figure 3-7).

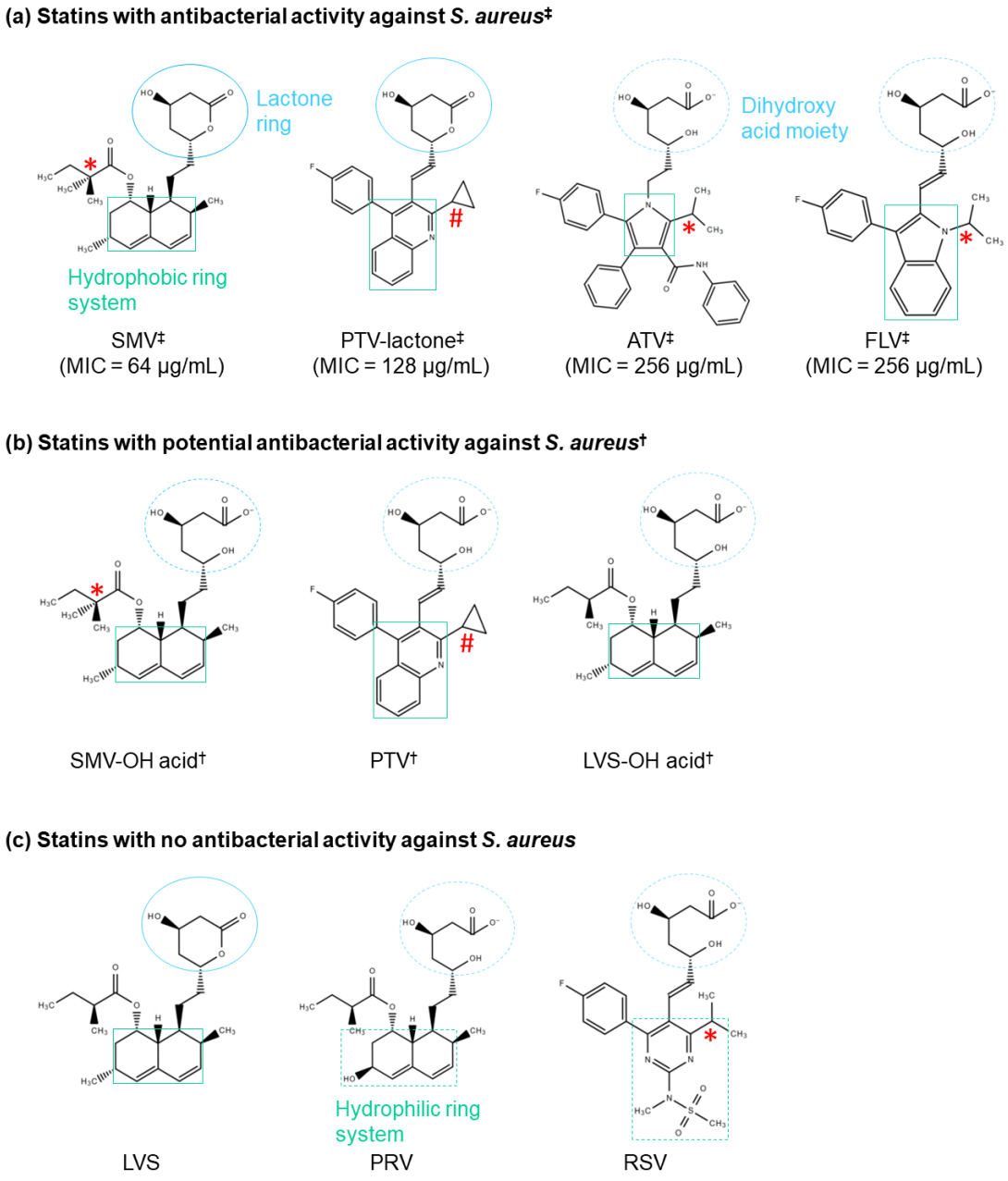

Figure 3-7: Structure-activity relationship analysis to identify functional groups responsible for antibacterial activity against *S. aureus*.
(a) (‡) Statins with antibacterial activity against *S. aureus*, whereby MIC was determined visually with the unaided eye. **(b)** (†) Statins with potential antibacterial activity against *S. aureus* (statistically significant antibacterial effects were indiscernible to the unaided eye but detected via spectrophotometry). **(c)** Statins with no antibacterial activity against *S. aureus*. (*) A *gem*-dimethyl moiety (two methyl groups on the same carbon atom) with a tetrahedral molecular geometry. (#) A cyclopropyl ring. Lactone rings are marked with solid ovals; dihydroxy acid moieties with dotted ovals; hydrophobic ring systems with solid rectangles; and hydrophilic ring systems with dotted rectangles. The combined presence of a hydrophobic ring system, lactone ring, and (*) or (#) likely confers greatest antibacterial activity (SMV versus SMV-OH acid, or PTV-lactone versus PTV). A hydrophilic ring system likely reduces antibacterial activity (PRV versus LVS-OH). [Reprinted with permission from Springer, Eur J Clin Microbiol Infect Dis.[130] Copyright (2018)]

A hydrophobic ring system might be a prerequisite for antibacterial activity as the presence of a hydrophilic ring system does not appear to impart activity (PRV and RSV) but may instead, reduce antibacterial activity (PRV versus LVS-OH acid). SMV and LVS differ by only one methyl group in the ester side chain, yet SMV exerted antibacterial activity but LVS does not (Figure 3-7). This suggests the importance of an extra methyl group, specifically from a *gem*-dimethyl moiety.

The lactone ring alone without a *gem*-dimethyl moiety or a cyclopropyl ring (LVS) does not confer antibacterial activity (Figure 3-7). A dihydroxy acid moiety and a *gem*-dimethyl moiety (or cyclopropyl ring) in a hydrophobic ring system may contribute activity (ATV and FLV) or potential activity (SMV-OH acid and PTV), but the effect is not as significant as when a lactone ring is present instead (SMV versus SMV-OH acid, or PTV-lactone versus PTV). Dihydroxy acid moiety combined with a *gem*-dimethyl moiety in a hydrophilic ring system however, did not demonstrate activity (RSV). The dihydroxy acid moiety alone without a *gem*-dimethyl moiety or a cyclopropyl ring in a hydrophobic ring system may present potential antibacterial activity (LVS-OH versus LVS), but not when alone in a hydrophilic ring system (PRV).

3.4.3 Postulated mechanism of antibacterial activity

Bacteria may attach to environmental surfaces through non-polar interactions between a methyl group and an alanine residue.[140] A cyclopropyl ring may also bind with an alanine residue through hydrophobic interactions.[141] Wall teichoic acids and lipoteichoic acids are structures which protrude from Gram-positive bacteria cell membranes and contain alanine residues.[142] Therefore, we hypothesise that statin's antibacterial activity may involve the interaction of a methyl group from the *gem*-dimethyl moiety (SMV, ATV, or FLV) or cyclopropyl ring (PTV-lactone) with the alanine residues of lipoteichoic acids from Gram-positive bacteria through van der Waals forces or hydrogen bonding.[143] This may cause structural distortions of the lipoteichoic acids (resulting in cell division interference),[144] or decrease the number of available alanine residues (thus reducing biofilm formation and bacterial adhesion to environmental surfaces).[142]

Several other observations, when viewed collectively, support our hypothesis. There are also other surface proteins responsible for various roles in *S. aureus* such as adhering to and invading host cells, evading host immune responses, and formation of biofilms.[145] Statins are able to change their conformation and bind extensively to proteins (≥ 88% protein binding, except for PRV which exhibits about 43% to 54% protein binding) through van der Waals forces and hydrogen bonds.[38, 146] Therefore, the binding of statins to bacterial surface proteins may influence various metabolic pathways to reduce bacteria proliferation and virulence. This might account for the lack of antibacterial activity of PRV, which possessed significantly lower protein binding properties.

Propranolol (an antihypertensive) with a *gem*-dimethyl moiety also demonstrated antibacterial activity against *S. aureus*.[147] The $MIC_{[SMV]}$ for MRSA is higher than $MIC_{[SMV]}$ for MSSA.[42] Since MRSA cocci are smaller and have higher cell surface to plasma ratio compared to MSSA cocci,[148] more SMV may be required to bind to the greater number of teichoic acid surface structures in MRSA, compared to MSSA cocci.

Adding exogenous cholesterol to Gram-positive bacteria decreased the antibacterial effects of statins.[76] Since *S. aureus* can integrate exogenous cholesterol into its membrane,[149] the resultant increase in cell membrane rigidity may prevent statins from binding to or distorting cell surface structures.

3.4.4 Limitations of study

Our study had two main limitations, namely the inability to distinguish the impact of undissolved SMV particles on MIC results, and the inability to attain actual concentrations of 256 μg/mL and 128 μg/mL due to insolubility of SMV at these concentrations, which also limited the ability to determine a minimum bactericidal concentration for SMV.

Although SMV at 64 μg/mL exerted antibacterial activity against *S. aureus*, we could not assume similar antibacterial effects at higher SMV concentrations. The incomplete dissolution of SMV at 256 μg/mL and 128 μg/mL before and after incubation (Figure 3-6a) introduced an additional variable (undissolved drug

particles), which could influence MIC results through plausible interactions with the broth, bacteria, and/or dissolved drug particles during incubation. We could not increase the solubility of SMV via increasing the solvent concentration (high concentrations of methanol may exert antibacterial effects), or changing conditions such as pH or temperature (regulated by CLSI guidelines). In addition, we decided not to use bacterial tracers, as these would also introduce additional variables such as chemical or physical interactions with the broth, bacteria, solvent, dissolved, or undissolved drug.

The method described in Section 3.2.6 allowed us to determine that the turbidity at 256 μg/mL and 128 μg/mL after incubation was attributed to both undissolved drug and bacterial growth (Figure 3-6). However, we could not distinguish if the undissolved SMV contributed to bacterial growth, for example, via physically protecting bacteria within flocculated undissolved drug particles, allowing bacteria to thrive. Conversely, if our results showed inhibition of bacterial growth at these concentrations, we would not be able to distinguish if the undissolved drug contributed to the antibacterial activity.

The SMV concentrations labelled as "256 μg/mL" and "128 μg/mL" in our study effectively contained less dissolved drug than labelled because these wells contained excess undissolved drug particles before and after incubation for 20 hours (Figure 3-6a). The saturated concentration of SMV before incubation was slightly less than 64 μg/mL, since at this concentration, SMV appeared visually clear but slight turbidity was detected by the spectrophotometer (Figure 3-6c, OD625 was less than 20% at 0 hours for SMV = 64 μg/mL). With the care taken during dilution and verification of similar OD625 amongst wells with the same concentration as described above (Sections 3.2.2 and 3.2.3), we could be reasonably assured that the actual concentration of the wells with undissolved SMV before incubation would be above the saturation concentration (approximately 64 μg/mL) but not higher than the respective 256 μg/mL and 128 μg/mL concentrations at which they were labelled.

Despite these limitations, our MIC result (64 μg/mL) for SMV is still valid, which also revealed *S. aureus* exhibited a paradoxical growth effect, whereby SMV inhibited bacterial growth more effectively at a lower drug concentration (64 μg/mL)

rather than at higher drug concentrations (128 μg/mL or 256 μg/mL) (Figure 3-6). A paradoxical growth effect occurs when greater antimicrobial activity is exhibited at lower drug concentrations instead of higher concentrations.[150] This anomaly is usually observed *in vitro*, and likely specific to the microorganism strain, species, and type of drug used.[150] It is more pronounced for high protein binding drugs in culture media without albumin.[151] Explanations for this phenomenon include drug insolubility at high concentrations; biofilm formation increasing antimicrobial resistance; activation/inactivation of certain metabolic pathways or resistance mechanisms attenuating antimicrobial effects; or programed altruistic death of bacteria at sufficiently high antibiotic concentrations resulting in cell lysis and release of materials to aid growth of other cells.[151-154]

This anomaly was also observed in another study when *S. aureus* ATCC 29213 (same strain used in this study) was tested in a different media without albumin (tryptic soy broth), utilised SMV from a different supplier, and was completely dissolved by a different solvent (DMSO).[64] Although it was not specifically discussed, the results of Wang et al. showed that after 8 hours of incubation, bacterial density of SMV at 62.5 μg/mL was lower than at 125 μg/mL, and continued to be so when extrapolated to 20 hours of incubation as recommended by the CLSI guidelines.[64, 67] Hence, a paradoxical growth phenomenon is plausible for *S. aureus* exposed to SMV in albumin-free culture media, despite utilising SMV from a different source or using a different solvent.

Future laboratory research to confirm whether a paradoxical growth effect exists could involve reviewing the optimal antibacterial dose for SMV and simulating physiological conditions by supplementing culture media with human serum albumin (which may reduce the impact of the paradoxical phenomenon).[151] The high protein binding (> 95%) properties of SMV and albumin-free CAMHB media could have amplified this phenomenon.[151] Biofilm formation might not be a contributing factor to the paradoxical effect because although methanol as a solvent could have enhanced biofilm formation,[138] SMV has been shown to reduce *S. aureus* biofilm formation and viability.[60] Although the roles of specific metabolic pathways, resistance mechanisms, or programed altruistic cell death have also been proposed as

plausible explanations for the paradoxical growth phenomenon, this study is unable to categorically support any these aforementioned mechanisms.

3.5 Conclusions

The repurposing of SMV and PTV-lactone as topical antibacterial agents for *S. aureus* infections may be feasible as both drugs exerted the greatest bacteriostatic effects out of all the statins tested in this study. None of the tested statins demonstrated significant antibacterial activity against the selected Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *S. marcescens*) which may cause complicated SSTIs. However, spectrophotometry revealed that SMV-OH acid could be active against *S. aureus*, *E. coli*, and *S. marcescens* at higher drug concentrations (> 256 μg/mL).

A paradoxical growth phenomenon was observed when SMV inhibited *S. aureus* growth at a lower drug concentration (64 μg/mL) rather than at higher concentrations (128 μg/mL or 256 μg/mL), which could theoretically result in therapeutic failure at high drug concentrations. Through structure-activity relationship analysis, we postulate that statins' antibacterial action may involve statins binding with alanine residues of teichoic acids present on Gram-positive bacterial cell surfaces via the combination of a hydrophobic statin ring system, a lactone ring moiety, and a *gem*-dimethyl moiety or a cyclopropyl ring. Such interactions could disrupt teichoic acid structures or decrease the number of alanine residues, resulting in reduced biofilm formation, diminished bacterial adhesion to environmental surfaces, or impeded *S. aureus* cell division.

For future research, the use of up to 10% DMSO may confer several clinical advantages over methanol as a solvent for water-insoluble statins. Further studies are also necessary to assess the safety of utilising high statin doses topically, especially when combined with other antibiotics to treat SSTIs such as fluoroquinolones and macrolides, which are known to increase SMV concentrations.

Having demonstrated laboratory evidence of statins as a plausible novel topical antibiotic for SSTIs due to MSSA infections, the next step in the proposed

translational research framework involved evaluating applied research in clinical practice, which involves determining the effects of statins in patients with SSTIs.

CHAPTER FOUR

4. Ambulatory Care Evidence (Sequence Symmetry Analysis)

4.1 Preamble

It has been reported that statins may reduce the risk of community-acquired *S. aureus* bacteraemia and exert antibacterial effects against *S. aureus*.[155] Together with the results of SMV and PTV-lactone demonstrating direct antibacterial activity in the previous chapter, it would be reasonable to hypothesise that statins could lower the risk of SSTIs or evolve into promising novel treatments for SSTIs.

However, statins may also induce new-onset diabetes mellitus ("diabetes mellitus" referred as "diabetes hereafter),[129] which is a risk factor for SSTIs.[13] Additionally, skin colonisation with *S. aureus* predisposes diabetic patients to infections,[156] as well as recurrent SSTIs.[11, 157] By inhibiting HMG-CoA reductase, statins reduce cholesterol production, but the inhibition of epidermal cholesterol synthesis may compromise the skin's barrier function,[158] paradoxically raising the risk of SSTIs. Furthermore, it has been suggested that the observed benefits of statins with respect to infections might be a result of a "healthy user effect", whereby statin users were more likely motivated to engage in healthy lifestyles, hence resulting in a biased positive effect.[159]

Given the above plausible yet conflicting theories, the work in this chapter sought to determine whether statins manifested a beneficial or detrimental clinical outcome in outpatients with SSTIs by evaluating the interrelationships between statins, diabetes, and SSTIs.

This chapter was initially submitted as a manuscript entitled "A sequence symmetry analysis of the interrelationships between statins, diabetes, and skin infections" for consideration of publication in the peer-reviewed *Medical Journal of Australia* but it was not accepted. The manuscript was subsequently resubmitted to another peer-reviewed journal (*British Journal of Clinical Pharmacology*) and recently accepted for publication on 8th October 2019.[160] This is the peer reviewed version of the following article "A sequence symmetry analysis of the interrelationships between statins, diabetes and skin infections. Br J Clin Pharmacol. 2019; 85(11):2559-2567",

Relevant parts of the original manuscript have been edited and presented in this chapter from Section 4.2 onwards to facilitate flow of the thesis. The labels for references and figures have been amended to align with the thesis format. The abstract and introduction sections of the original manuscript have been abridged and adapted in this preamble. The methods, results, and discussions have been expanded in this chapter due to a word limit for the original article. The original conclusion has been revised in this thesis to promote transition between chapters.

All authors had no competing interests to declare. The primary investigator performed the literature and reference searches, collected the data, prepared the figures and tables, wrote the manuscript, and contributed significantly to the design, analysis, and interpretation of findings as lead author in the peer-reviewed publication. Permission was obtained from all co-authors to include the contents of the published article for this thesis (Appendix 8). Ethics approval (E014/003; Appendix 9) has been granted by the Australian Department of Veterans' Affairs (DVA).

4.1.1 Objectives

This work aimed to determine statins' impact on outpatients with SSTIs, taking into consideration that statins might reduce the risk of *S. aureus* infections, but may also paradoxically increase SSTI risks due to statins' association with new-onset diabetes, a risk factor for SSTIs.

The SSA was chosen for this study, which served as a self-controlled design in pharmacoepidemiology.[161] The interrelationship between statins, diabetes, and SSTIs were segregated into the three possible pairs (statins-SSTIs, statins-diabetes, and diabetes-SSTIs), and SSA was performed to ascertain if: [i] statins increased the risk of SSTIs; [ii] statins increased the risk of diabetes; and [iii] diabetic patients were

susceptible to SSTIs. The results from these three analyses would identify if each pair exerted a beneficial or detrimental clinical outcome. Collectively, they corroborate the likely association of statins and SSTIs.

A secondary analysis on the influence of probable healthy user effects was also conducted for each of the studied pairs, using socio-economic status as a surrogate indicator, since the healthy user bias was closely aligned with socio-economic welfare.[162]

4.1.2 Potential significance of the research

By analysing a large database of prescriptions from the Australian DVA spanning over more than 10 years, the time taken to exhibit possible associations could be ascertained for each of the pairs studied (statins-SSTIs, statins-diabetes, and diabetes-SSTIs). This provides clinicians with useful information on the sensitive period, a time frame in which exposure to an event may be associated with the greatest risk of disease development.[163]

The secondary analysis on socio-economic status serves to indicate whether the healthy user effect played a significant role in influencing the results for each pair studied.

4.2 Methods

The SSA was originally used as an economical and rapid means of reviewing adverse drug reactions using prescription drugs.[1] The analysis was later expounded,[164] and has since gained popularity in pharmacoepidemiology to detect adverse events.[1] Advantages of the SSA over other epidemiological study designs include controlling for confounding factors which do not vary considerably over the study period, such as age, gender, or genetics.[1, 165]

To detect adverse events using SSA, the sequence of incident (first-time) prescriptions of patients taking both the drug of interest (index drug) and the drug specifically indicated for treating the adverse event (marker drug) is examined.[164, 165] Prescription sequences with intervals greater than 365 days between the index and

marker drugs were not analysed to minimise potential time-varying confounders such as age. If the index drug increases the probability of an event, the number of incident index drugs prescribed first ($n_{\mathbf{index \rightarrow marker}}$) will be expected to be significantly larger than the number of incident marker drugs prescribed first ($n_{\mathbf{marker \rightarrow index}}$). The crude sequence ratio (CSR) of incident prescriptions ($n_{\mathbf{index \rightarrow marker}}$)/($n_{\mathbf{marker \rightarrow index}}$) will thus be greater than unity. The fundamental assumption for this analysis is that if there was no causal association, incident users of both the index and marker drugs follow similar incidence trends for each drug in the study population.[165]

Incident prescribing trends may vary over time. Hence a null-effect sequence ratio (NSR), the expected sequence ratio in the absence of any causal relationship, is calculated to adjust for these trends (Appendix 10).[164, 165] The adjusted sequence ratio (ASR), calculated as CSR/NSR, is the incidence rate ratio of marker drug prescribing in index drug exposed versus non-exposed person-time.[164] Since the variance of the NSR is negligible compared to the variance of the CSR (which is much larger), the confidence interval (CI) of ASR is therefore largely determined by the CI of the CSR and calculated using the binomial distribution and crude number of sequences.[164]

4.2.1 Data source

Permission was obtained from DVA to study prescription claims made by over 228,000 veterans, war widows, and widowers from 1st January 2000 to 31st December 2012.[166] Prescriptions filled for statins (ATV, FLV, PRV, RSV, and SMV), antidiabetic medication (insulins, insulin analogues, and oral blood glucose lowering drugs; Appendix 11), and antistaphylococcal antibiotics (dicloxacillin and flucloxacillin) were examined using non-identifiable client numbers, dates of prescriptions filled, residential electorates, and Anatomical Therapeutic Chemical codes as defined by the World Health Organization (Appendix 11).[167]

4.2.2 Primary analysis

A waiting-time distribution graph of the total number of all first-time prescriptions filled was plotted from 1st January 2000 to 31st December 2012 to determine the run-in period, which was the initial short time frame containing both incident users (first-time prescription claims which are relevant for analysis) and prevalent users (repeat prescription claims which are not relevant for analysis).[164, 165] By excluding the run-

in period from the study, the later remaining time frame would be the study period which consists of only incident users (the population of interest).

Thereafter, SSA was performed on first-time prescription data from the study period (after the run-in period) to determine if: [i] statins increased risk of SSTIs ($index_{[statins]}$; $marker_{[antistaphylococcal\ antibiotics]}$); [ii] statins increased risk of diabetes ($index_{[statins]}$; $marker_{[antidiabetic\ medication]}$); and [iii] diabetic patients were susceptible to SSTIs ($index_{[antidiabetic\ medication]}$; $marker_{[antistaphylococcal\ antibiotics]}$) (Figure 4-1).

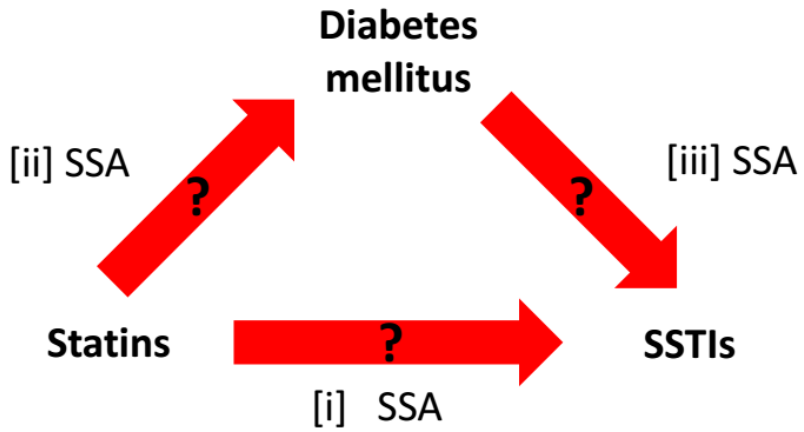

Figure 4-1: Using SSA to evaluate plausible interrelationships between statins, diabetes mellitus, and SSTIs.
[i] Between statins and SSTIs, index drug = statin, marker drug = antistaphylococcal antibiotics. **[ii]** Between statins and diabetes, index drug = statin, marker drug = antidiabetic medication. **[iii]** Between diabetes and SSTIs, index drug = antidiabetic medication, marker drug = antistaphylococcal antibiotics. Statins included ATV, FLV, PRV, RSV, and SMV; antidiabetic medication included insulins, insulin analogues, and oral blood glucose lowering drugs (Appendix 11); and antistaphylococcal antibiotics included dicloxacillin and flucloxacillin. [Reprinted with permission from John Wiley and Sons, Br J Clin Pharmacol.[160] Copyright (2019)]

The SSA was performed at window intervals of 91, 182, and 365 days for each relationship to identify variations in risk over time. For example, if statins are associated with an increased risk of SSTIs (Figure 4-1, direction [i] favoured) within 91 days of statin use, the number of statins (index drug) prescribed first ($n_{\textbf{statins first}\rightarrow \textbf{antistaphylococcal antibiotics second}}$) will be expected to be significantly larger (ie. more people requiring antistaphylococcal antibiotics after taking statins) than the number of antistaphylococcal drugs (marker drug) prescribed first ($n_{\textbf{antistaphylococcal antibiotics}}$

first→statins second) over any 91-day time frame. The CSR of incident prescriptions ($n_{\mathbf{index\rightarrow marker}}$)/($n_{\mathbf{marker\rightarrow index}}$) and subsequently calculated ASR will thus be greater than unity. This analysis was repeated for any 182-day and 365-day time frames within the study period, and similar analyses were conducted for directions [ii] and [iii] as shown in Figure 4-1.

4.2.3 Confirmatory analysis

Amongst all first-time statin users in the study period, additional SSA was performed on diabetics (taking antidiabetic medication) and non-diabetics (not taking antidiabetic medication) to determine if statins contributed to the risk of SSTIs independently, regardless of diabetes status.

4.2.4 Secondary analysis

The Index of Relative Socio-economic Advantage and Disadvantage (IRSAD) provides a snapshot of the socio-economic status of inhabitants within a residential area in Australia.[168] A low or high score suggests that residents are generally disadvantaged or advantaged respectively, with the overall average score being 1006.[168] By charting the number of patients with known residential electorates (at time of filling first prescriptions) against IRSAD scores, the graph gives an overview of whether socio-economic status influences the proportion of ($n_{\mathbf{index\rightarrow marker}}$) patients against ($n_{\mathbf{marker\rightarrow index}}$) patients.

4.2.5 Statistical analysis

Data were analysed using SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA) and graphs drawn with GraphPad Prism version 7 (GraphPad Software, La Jolla, California, USA).

4.2.6 Ethics approval

This study was approved by the DVA Ethics Committee (E014/003, Appendix 9).

4.3 Results

4.3.1 Primary analysis

From the waiting-time distribution graph (Figure 4-2), a run-in period of six months was required to exclude prevalent users. Our study period was hence from 1st July 2001 to 31st December 2011 inclusive, to allow the analysis of the 365 days window interval preceding the first drug prescribed, and 365 days window interval following the last drug prescribed (Figure 4-2).

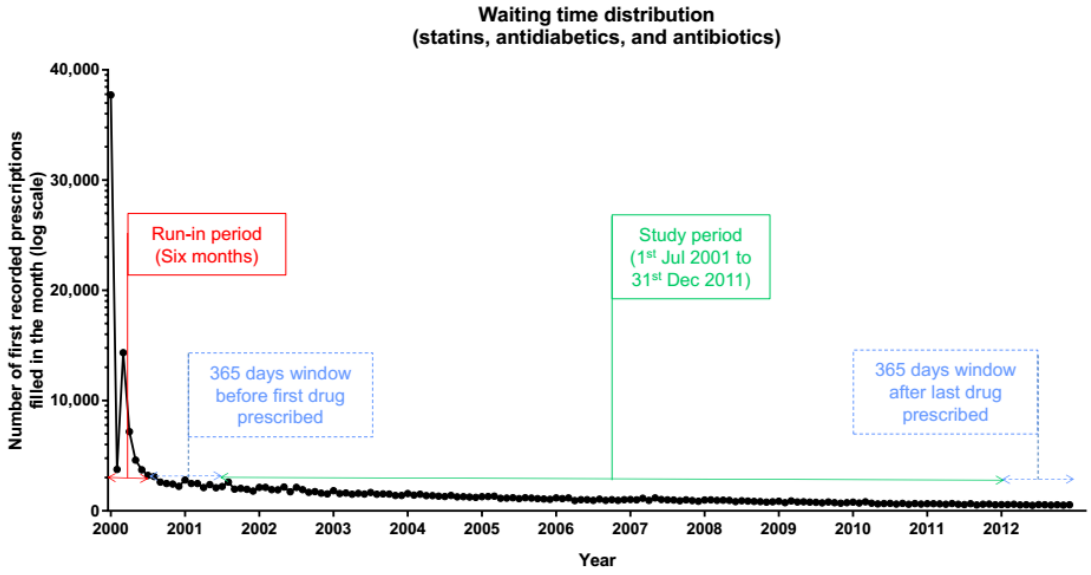

Figure 4-2: Waiting time distribution graph for all drugs (statins, antidiabetics, and antibiotics) involved in this study.
The run-in period, time taken to differentiate incident users (evenly distributed over time) from prevalent users (clustered at initial phase of study), was identified as six months. Hence the effective study period was from 1st July 2001 to 31st December 2011.

Overall, statins were associated with a significant risk of SSTIs. This risk was similar over 91, 182, or 365 days (Figure 4-3: ASR = 1.40, 1.41, and 1.40 respectively; CI > 1), with the greatest influence from ATV and SMV (Figure 4-3).

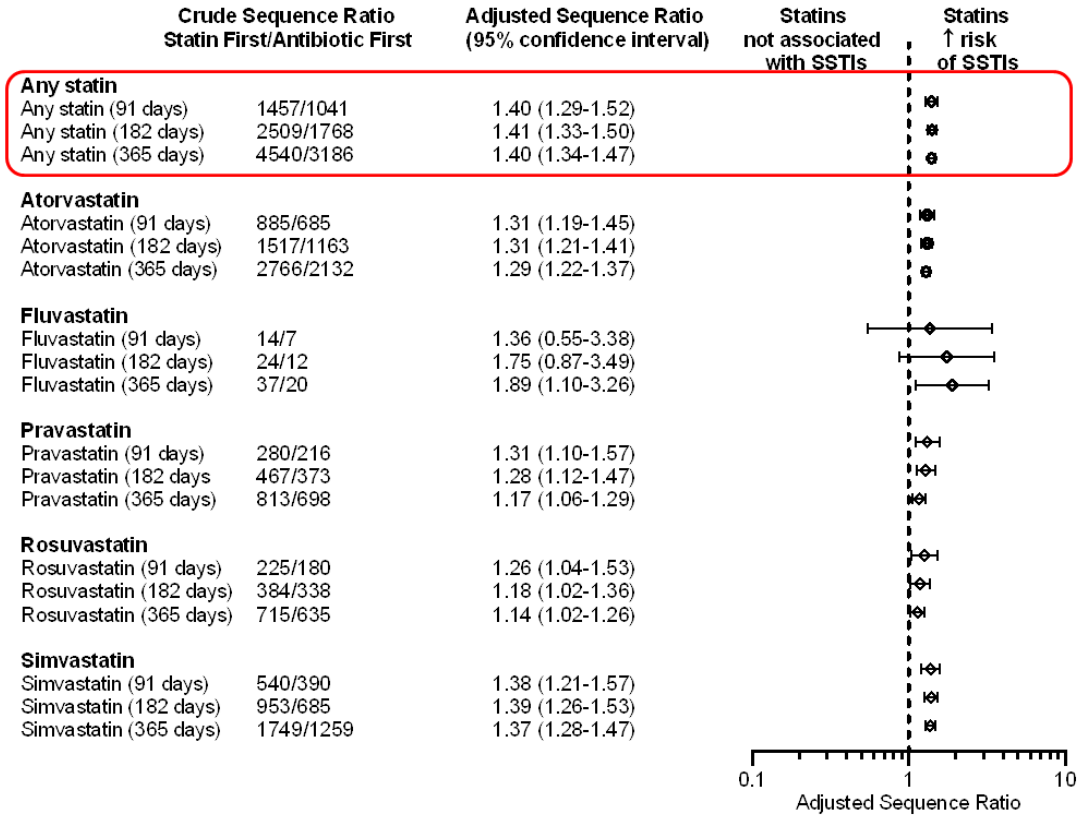

Figure 4-3: Results of SSA for the relationship between statins and SSTIs. Index drugs used were statins (ATV, FLV, PRV, RSV, and SMV). Marker drugs used were antistaphylococcal antibiotics (dicloxacillin and flucloxacillin). Number of records for "any statin" will either be equal to or less than the summation of records for individual statins because two or more individual statins presented on the same day would still be considered as one record under the "any statin" analysis. [Reprinted with permission from John Wiley and Sons, Br J Clin Pharmacol.[160] Copyright (2019)]

Statins were also associated with a significant risk of new-onset diabetes, but the risk decreased gradually over 91, 182, and 365 days (Figure 4-4: ASR = 1.19, 1.14, and 1.09 respectively; CI > 1). ATV and SMV were also the greatest contributors to this outcome, albeit the results were not statistically significant over 365 days (Figure 4-4).

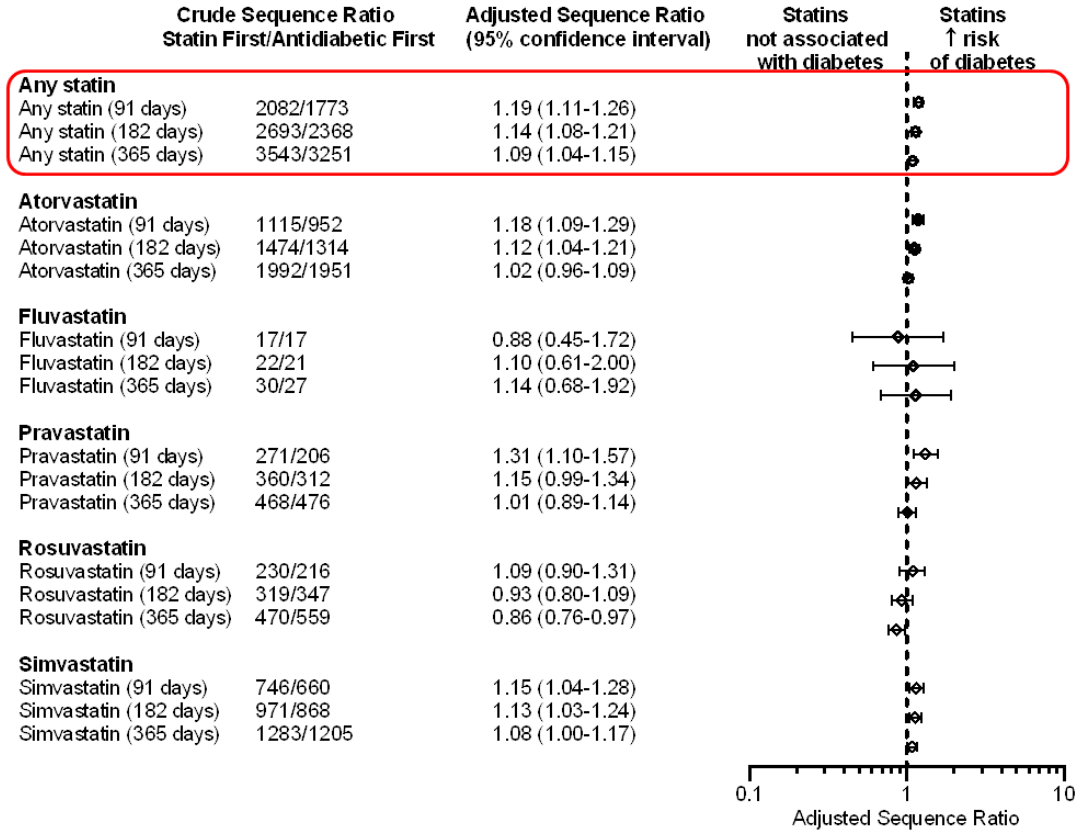

Figure 4-4: Results of SSA for the relationship between statins and diabetes mellitus.
Index drugs used were statins (ATV, FLV, PRV, RSV, and SMV). Marker drugs used were antidiabetic medication (insulins, insulin analogues, and oral blood glucose lowering drugs). Number of records for "any statin" will either be equal to or less than the summation of records for individual statins because two or more individual statins presented on the same day would still be considered as one record under the "any statin" analysis. [Reprinted with permission from John Wiley and Sons, Br J Clin Pharmacol.[160] Copyright (2019)]

Patients with diabetes were associated with increased risk of SSTIs at the 182 and 365 days window (Figure 4-5: ASR = 1.20 and 1.24 respectively, CI > 1 respectively), but the risk was non-significant at the 91 days window (Figure 4-5: ASR = 1.14; CI overlaps unity).

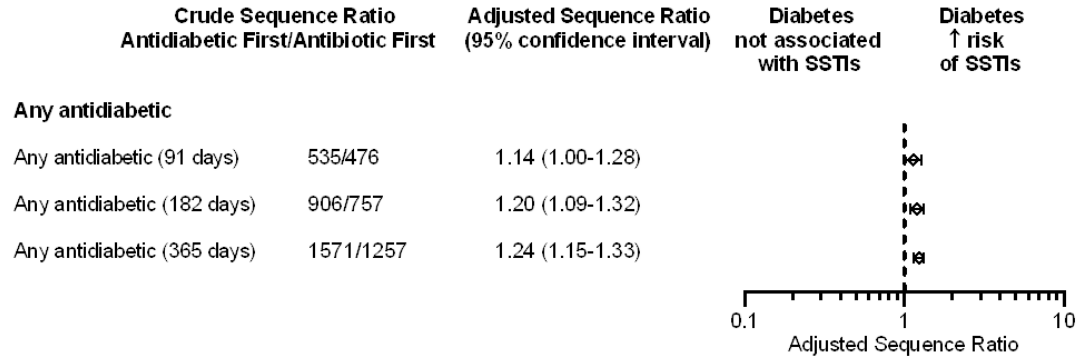

Figure 4-5: Results of SSA for the relationship between diabetes mellitus and SSTIs.
Index drugs used were antidiabetic medication (insulins, insulin analogues, and oral blood glucose lowering drugs) as listed in Appendix 11. Marker drugs used were antistaphylococcal antibiotics (dicloxacillin and flucloxacillin). [Reprinted with permission from John Wiley and Sons, Br J Clin Pharmacol.[160] Copyright (2019)]

4.3.2 Confirmatory analysis

Non-diabetic statin users were found to have significant risk of SSTIs at 91, 182, and 365 days (Figure 4-6: ASR = 1.39, 1.41, and 1.37 respectively, CI > 1 respectively). Diabetic statin users were similarly shown to be at significant risk of SSTIs at 91, 182, and 365 days (Figure 4-6: ASR = 1.43, 1.42, and 1.49 respectively, CI > 1 respectively).

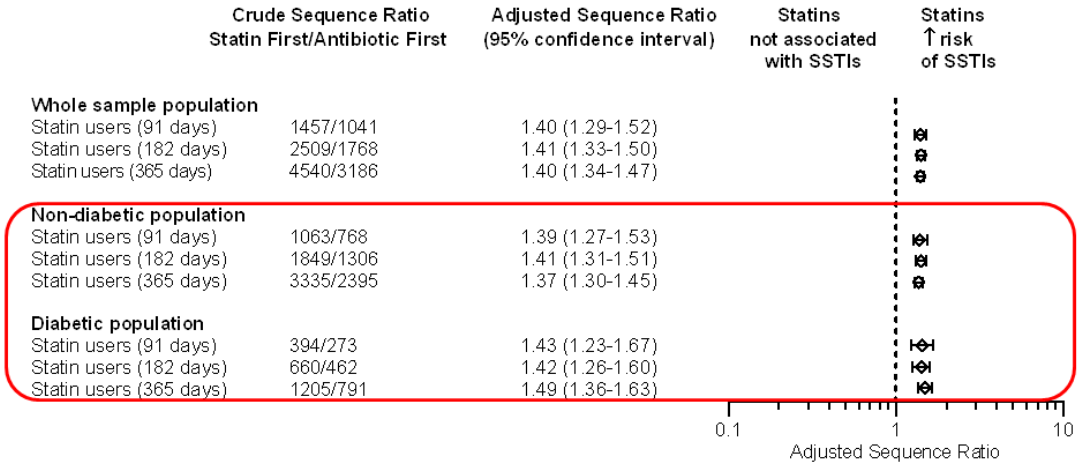

Figure 4-6: Confirmatory sequence symmetry analysis to determine the risk of SSTIs associated with non-diabetic statin users compared to diabetic statin users.
Diabetic population was defined as patients on antidiabetic medication (insulins, insulin analogues, and oral blood glucose lowering drugs). Index drugs used were statins (atorvastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin). Marker drugs used were antistaphylococcal antibiotics (dicloxacillin and flucloxacillin). [Reprinted with permission from John Wiley and Sons, Br J Clin Pharmacol.[160] Copyright (2019)]

4.3.3 Secondary analysis

The proportion of ($n_{\mathbf{index \rightarrow marker}}$) patients to ($n_{\mathbf{marker \rightarrow index}}$) patients with relatively disadvantaged (IRSAD < 1006) and advantaged (IRSAD > 1006) socio-economic conditions did not differ significantly for: [i] statin and antibiotic users (p = 0.716; Figure 4-7i); [ii] statin and antidiabetic users (p = 0.07; Figure 4-7ii); and [iii] antidiabetic and antibiotic users (p = 0.94; Figure 4-7iii).

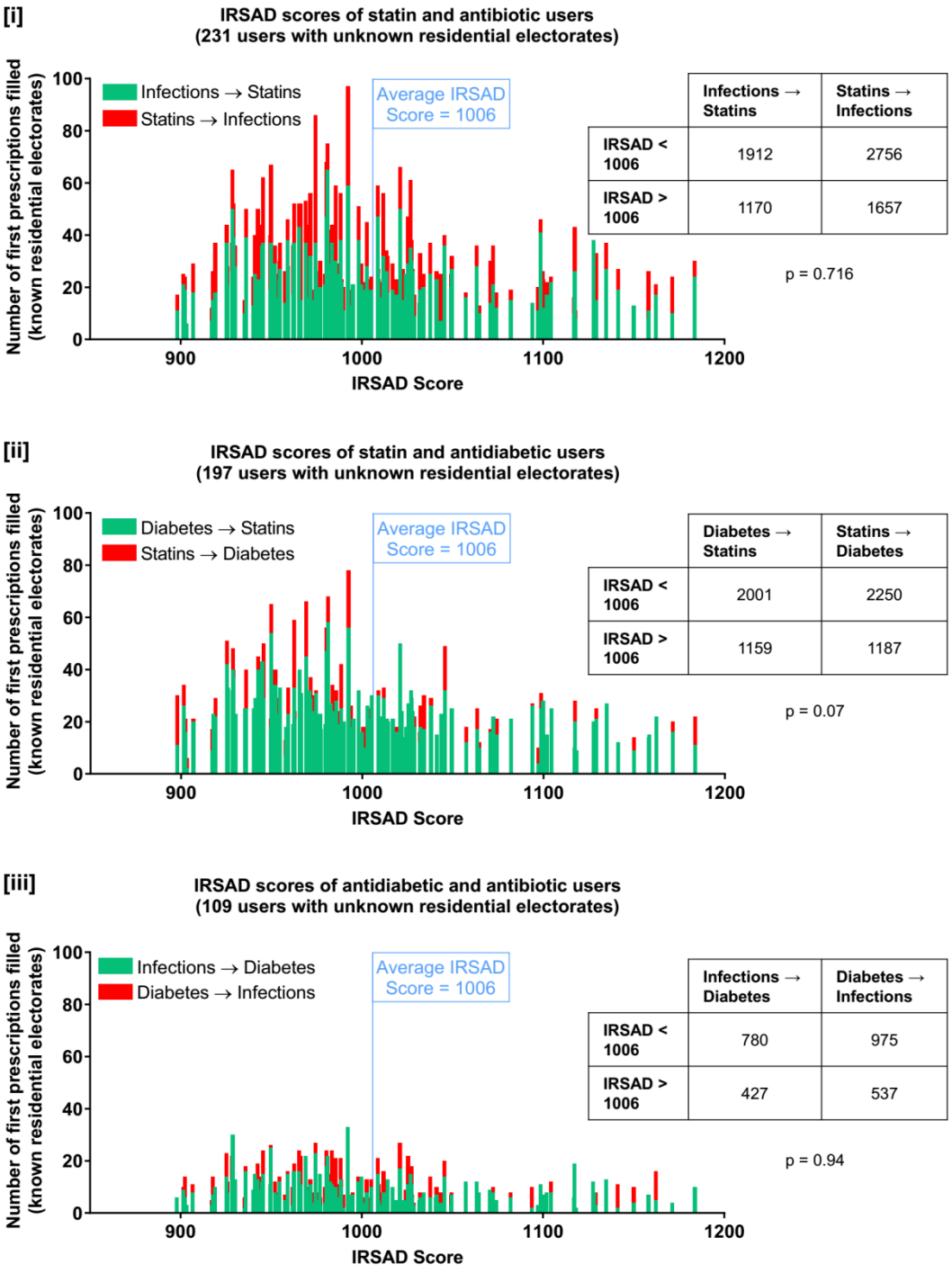

Figure 4-7: Index of Relative Socio-economic Advantage and Disadvantage (IRSAD) scores reflecting socio-economic status of patients (with known residential electorates) who filled first prescriptions.
Chi-square tests were performed for each of the three groups to detect significant differences (if $p < 0.05$) in the proportion of ($n_{\text{index}\rightarrow\text{marker}}$) patients to ($n_{\text{marker}\rightarrow\text{index}}$) patients with relatively disadvantaged (IRSAD < 1006) and advantaged (IRSAD > 1006) socio-economic conditions. **[i]** Relationship between statins and skin infections: index drug = statin, marker drug = antistaphylococcal antibiotics. **[ii]** Relationship between statins and diabetes: index drug = statin, marker drug = antidiabetic medication. **[iii]** Relationship between diabetes and SSTIs: index drug = antidiabetic medication, marker drug = antistaphylococcal antibiotics.

4.4 Discussion

To our knowledge, there are currently no known clinical studies of statins specifically associated with the risk of SSTIs. However, there are conflicting conclusions about the effect of statins on the risk of general infections, some supporting statins reducing the risk of infections,[155, 169] while others refute this beneficial outcome.[170, 171] By reconciling our results with available literature that utilise non-SSA related methodologies, clinical outcomes which align with our results would support plausible mechanism(s) of action for statins in SSTIs and diabetes.

4.4.1 Statins and risk of SSTIs

Current clinical literature supports direction [ii] of Figure 4-1 (statins being associated with diabetes),[129, 172] as well as direction [iii] of Figure 4-1 (diabetes being associated as a risk factor of skin infections).[8, 13] Our results showed that statin users were associated with an increased risk of SSTIs (Figure 4-3), as well as an increased risk of diabetes (Figure 4-4), and diabetes was associated with an increased risk of SSTIs (Figure 4-5).

The confirmatory analysis revealed that both non-diabetic and diabetic statin users were associated with similar significantly increased risks of SSTIs (Figure 4-6). Diabetes is a risk factor for SSTIs in non-statin users, since diabetes has been shown to increase the risk of general infections,[173] as well as specifically skin infections.[8, 13] As such, without influence from extraneous factors, it would be reasonable to expect non-diabetics (regardless of statin use) to have low to no risk of SSTIs. However, the confirmatory analysis showed that both non-diabetic and diabetic statin users had similar significantly increased risks of SSTIs, alluding to statin use as an important contributor to SSTI risk. Viewed collectively, it may be posited that statins are associated with an increased SSTI risk, whether indirectly (via diabetogenic mechanisms) (Figure 4-1, directions [ii] and [iii]), or directly (via non-diabetogenic mechanisms) (Figure 4-1, direction [i]).

The findings of this study were in contrast to those reported by Pouwels et al.,[174] who reported a reduction in antibiotic use in drug-treated type 2 diabetic statin users

compared to non-users. Although their research design also utilised SSA, they did not examine the effects of narrow spectrum antibiotics (such as dicloxacillin and flucloxacillin) which target mainly staphylococci, a major bacterial causative agent for SSTIs.[13] By studying all beta-lactam penicillins as a group,[174, 175] the effects of broad spectrum beta-lactam antibiotics on a variety of both Gram-positive and Gram-negative bacteria may mask or confound the results specific to Gram-positive staphylococci. Hence, it is possible that our results differed despite using the same methodology.

Interestingly, although the study by Liappis et al. concluded that statins may have a potentially therapeutic role in bacteraemic infections, they noted a statistically significant increase in SSTIs among patients with bacteraemia who were receiving statins, compared to those who were not using statins.[176] The work of both Liappis et al. (not designed *a priori* to detect an association between statins and SSTIs) and our study (designed *a priori* to detect this association) demonstrating the same outcome suggests the association between statins and SSTIs is unlikely to be spurious. The clinical evidence presented in the following two sections provide plausible mechanisms by which statin use could increase SSTI risk, whether via indirect (diabetogenic) mechanisms (Figure 4-1, directions [ii] and [iii]), or via direct (non-diabetogenic) mechanisms (Figure 4-1, direction [i]).

4.4.1.1 Statins and risk of diabetes (plausible indirect SSTI mechanism)

The diabetogenic mechanisms of statins may involve increased insulin resistance and/or diminished pancreatic β-cell function.[129] Patients with diabetes have impaired immunity, undermining the defence against pathogens such as *S. aureus*, hence increasing the risk of SSTIs.[20] Our study revealed that the sensitive period whereby statin exposure exerted the greatest risk, was within 91 days after statin commencement, especially for ATV and SMV (Figure 4-4). This suggests statin-induced diabetogenic mechanisms may be completed as soon as within 91 days.

The use of statins may upregulate low-density lipoprotein (LDL) receptors to reduce plasma LDL cholesterol (LDL-C), resulting in increased intracellular LDL-C burden and diminished pancreatic β-cell function.[129] In addition, the reduction of coenzyme Q10 as a result of mevalonate pathway inhibition may disrupt mitochondrial electron

transport and impair insulin secretion.[129] Clinical studies have shown that blood levels of LDL-C and coenzyme Q10 were reduced after daily doses of SMV (LDL-C ↓34.7%, coenzyme Q10 ↓31.2% after 28 days) and ATV (LDL-C ↓51%, coenzyme Q10 ↓52% after 30 days).[177] Since reduced plasma levels of LDL-C and/or coenzyme Q10 by statins are associated with an increased risk of diabetes,[129] it is conceivable for statin users (especially users of ATV and SMV, in alignment with Figure 4-4) to be at increased risk of diabetes after 30 days,[129, 177] and thereafter be at further risk of SSTIs over the next 60 days (in alignment with Figure 4-5) since diabetes is a risk for SSTIs.[13] As such, the reduction of LDL-C and/or coenzyme Q10 levels could be indirectly associated with an increased SSTI risk within 91 days of statin commencement via diabetogenic mechanisms (Figure 4-1, directions [ii] and [iii] and Figure 4-6).

Other studies utilising different research methods also supported the association of statins and diabetes in humans within time frames that aligned with this study. A study utilising pharmacometabolomics (quantification and analysis of metabolites produced by the body) reported that 40 mg of oral SMV daily for 6 weeks elevated the risk of increased plasma glucose.[178] A network meta-analysis of randomised clinical trials over 12 weeks to 12 month reported that compared to placebo, high-intensity ATV (dose range not specified) may exacerbate glycaemic control (increased glycated haemoglobin A1C and fasting plasma glucose levels), but moderate-intensity PTV may significantly improve glycaemic control in patients with type 2 diabetes.[179]

Disruption of the human gut microbiome, or gut dysbiosis, has been associated with the impaired metabolism of bile acids, which may impede glucose control and diminish innate immunity.[180] Bile acids regulate glucose homeostasis through the activation of nuclear receptors such as PXRs, and mount antimicrobial defences via activation of the vitamin D receptor.[108] Statins have been found to influence the human gut microbiome.[120] The clinical implications of this remains uncertain in our study, albeit remodelling of murine gut microbiota has been shown to increase the risk of diabetes in mice via PXR activation.[121]

A decrease in vitamin D levels may raise the risk of diabetes directly (via interference with insulin receptors, signalling, and glucose transport) or indirectly (secondary to hyperparathyroidism).[181] However, the overall effect of statins on vitamin D levels in humans is ambiguous. Statins decrease cholesterol (a precursor of vitamin D), which theoretically limits downstream vitamin D production. Yet, conflicting results revealed that statins may raise vitamin D levels (via competitive inhibition of the cytochrome P450 enzyme activity and activation of cholesterol membrane transporters to increase intestinal absorption of vitamin D),[182] as well as studies which showed that statins do not increase serum levels of vitamin D.[183]

The net effects of vitamin D on infections also appear inconclusive. Vitamin D may prevent infections by boosting the innate immunity (rapid response) through augmenting chemotaxis, phagocytosis, and activation of antimicrobial peptides.[184] However, by increasing T regulatory cells (Treg), and inhibiting T helper cell type 1(Th1) and type 17 (Th17),[184] the adaptive immune system (delayed response) against pathogenic infections may be dampened. Thus, the influence of vitamin D in this study is unclear.

4.4.1.2 Statins and the immune system (plausible direct SSTI mechanism)

The T helper cell types 1 (Th1) and 17 (Th17) are responsible for mounting the host's defence against pathogens, resulting in inflammatory responses.[185] The T regulatory (Treg) cells on the other hand, play a role in homeostasis by suppressing T cells, exerting anti-inflammatory effects.[185] Inhibition of HMG-CoA reductase by statins reduces cholesterol and downstream isoprenoids essential for intracellular signalling, which could result in the observed increase in anti-inflammatory Treg cells and decrease in pro-inflammatory Th1 and Th17 cells.[186] Clinical data show that statins inhibit the induction of Th1 and Th17 cells,[186] and may increase Treg cells within 4 to 12 weeks,[186, 187] time frames which corresponded with our SSA results demonstrating that statin users were associated with increased risk of SSTIs within 91 days (Figure 4-3). Given the importance of Th1, Th17, and Treg cells in skin immunity,[188] it is plausible that statin users may be directly associated with an increased SSTI risk within 91 days of statin commencement via non-diabetogenic mechanisms of reduced Th1, Th17 and increased Treg cell activities (Figure 4-1, direction [i] and Figure 4-6).

The skin functions as a crucial permeability barrier, providing innate immunity by protecting the host from noxious agents such as bacterial pathogens. Upon acute insult, epidermal cholesterol synthesis and HMG-CoA reductase activity increases swiftly to restore the protective barrier function.[158] Studies on mice have shown that topical application of statins impeded epidermal cholesterol synthesis and consequently, delayed recovery of the skin barrier function.[189] Additionally, high levels of cholesterol, in particular LDL-C, might confer immunoprotective effects against infections in mice.[190]

Since clinical studies have shown that plasma LDL-C could be reduced after about 30 days of ATV and SMV usage as discussed in Section 4.4.1.1,[177] and our SSA results demonstrated the use of ATV and SMV is associated with an increased risk of SSTIs within 91 days (Figure 4-3), the reduction of plasma LDL-C could also be a possible direct, non-diabetogenic mechanism by which statins are associated with increased SSTI risks. However, since this negative effect of cholesterol lowering on skin barrier function was demonstrated predominantly in mice, verification from clinical studies are required.

4.4.2 Healthy user effect

The "healthy user effect" refers to selective bias whereby motivated patients are more inclined to undertake preventive healthcare, such as consuming healthy diets and exercising frequently, and such health-seeking attitudes correspond closely with socio-economic status.[162] Since the residential electorate is reflective of patients' socio-economic status,[168] patients from electorates that are of above average IRSAD scores (> 1006) might be more likely than patients from below average IRSAD scores (< 1006) to exhibit traits such as reduced risk of infections or diabetes. However, the healthy user effect was not apparent because the role of socio-economic status was non-significant within the relationships examined (Figure 4-7).

4.4.3 Limitations of study

Due to the nature of SSA, patients were assumed to commence their medication on the day of filling their prescription and that they were compliant with medication, which might not have occurred in reality. We also assumed that all medicines were

administered as a Defined Daily Dose per day (Appendix 11),[167] thus we could not determine the impact of statin dosage on clinical outcomes.

Some antibiotics used to treat SSTIs may also be prescribed for other types of infections. By narrowing our choice of marker antibiotics to dicloxacillin and flucloxacillin, we could be reasonably assured that the data generated would be specific for bacterial SSTIs, albeit this excludes signals from the other antibiotics and precludes patients with penicillin allergies.

Confounding by indication is an inherent bias in SSA.[164] Since diabetes is a risk factor for SSTIs,[8, 13] an increased risk of SSTIs associated with statins could be confounded by an indication (diabetes) for taking statins. Diabetes is an important risk factor for cardiovascular diseases and statins are indicated in patients with diabetes to reduce the risk of cardiovascular diseases.[191] Hence, the number of patients ($n_{\text{antidiabetics 1st}\rightarrow\text{ statins 2nd}}$) may be relatively high, creating a bias towards an underestimation of statins' effect on diabetes, favouring the reverse of direction [ii] in Figure 4-1 and thereby, resulting in confounding by indication.

However, recommendations for statin prescribing to manage cardiovascular disease risks target metabolic syndrome, a condition comprising three of any of the following five factors: elevated waist circumference, elevated serum triglycerides, reduced HDL-C, elevated blood pressure, and elevated fasting glucose (diabetes).[191] As such, there are other conditions for prescribing statins which aim to control other components of metabolic syndrome but specifically exclude diabetes.[191] In these situations, ($n_{\text{statins 1st}\rightarrow\text{ antidiabetics 2nd}}$) would be relatively larger, favouring direction [ii] in Figure 4-1, which our results aligned with (Figure 4-4). Although we were unable to categorically rule out confounding by indication, our conclusion of stains being associated with diabetes via SSA methodology is supported by meta-analyses of randomised controlled trials.[192, 193]

Lastly, prescriptions for fixed-dose combination medicines have to be excluded from SSA studies because the CSR or ASR calculated using fixed-dose combination medicines could be attributed to any of the combined drugs, confounding the results generated. If the drugs were prescribed separately however, they could be in included

in SSA studies, boosting the sample size of drugs analysed. Although there is evidence that statins have been safe and efficacious when combined with other lipid-lowering drugs such as ezetimibe,[194] or antihypertensives such as amlodipine,[195] doctors tend to prescribe individual medicines for treating hypertension and hyperlipidaemia.[196] This could be due to guidelines for treating hypertension recommending angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) as first-line therapy over the combination medication containing amlodipine, a calcium-channel blocker.[197] Alternatively, a single statin at a higher dose may be sufficient for most patients in the prevention of cardiovascular diseases.[197] As such, the exclusion of fixed-dose combination medicines would unlikely cause a significant impact on the results obtained in this study.

4.5 Conclusions

Our study supports the hypothesis that first-time statin users are at increased risk of SSTIs and this risk was likely independent of diabetes status or the healthy user effect. Statins may directly increase SSTI risk via direct or indirect mechanisms. Clinical evidence with time frames that aligned with our results include the reduction of innate immunity via increase of Treg cells and inhibition of Th1 and Th17 cells within 91 days (direct, non-diabetogenic mechanism; Figure 4-1, direction [i]);[186, 187] and reduction of LDL-C and coenzyme Q10 levels within 91 days of statin commencement,[177] which increased the risk of diabetes, in turn a risk factor for SSTIs (indirect, diabetogenic mechanism; Figure 4-1, directions [ii] and [iii]).

Further clinical studies are required to confirm these mechanisms, as well as to ascertain the effect of statins on gut dysbiosis, impaired bile acid metabolism, reduced vitamin D levels, and cholesterol inhibition on skin function. Regardless of the actual mechanism(s), it would seem prudent for clinicians to monitor blood glucose levels of statin users who are predisposed to diabetes, and be mindful of possible increased risk of SSTIs in such patients.

Since statins may directly increase the risk of both SSTIs and diabetes, it appears the use of statins should ironically be avoided for patients with SSTIs. However, the results from this chapter do not include the study of PTV as it is currently not

registered in Australia for clinical use. The probable beneficial effect of PTV on blood glucose levels demonstrated by Cui et al.[179] has been supported by other studies,[198, 199] and this is of interest because its metabolite (PTV-lactone) demonstrated direct antibacterial effects as shown in Chapter Three of this thesis. The association between PTV and diabetes is being further investigated by other researchers in a randomised controlled trial,[200] and their results would help clarify if PTV-lactone has potential to be repurposed as an adjuvant/treatment for SSTIs.

The work done in the next chapter evaluated the effect of statins in patients hospitalised with SSTIs in Rockingham General Hospital, Western Australia. Although the effects of PTV were similarly not evaluated due to the drug being unregistered for clinical use in Australia, the work served to provide additional clinical evidence on the relationship between statin use and SSTIs.

CHAPTER FIVE

5. Hospital Care Evidence (Case-Control Study)

5.1 Preamble

Severe or unmanageable SSTIs at the outpatient setting would be better treated in the hospital, especially for complicated or necrotising infections which affect the deeper tissue layers. With the increasing emergence of antibiotic-resistant bacterial strains such as *S. aureus*, novel therapeutic agents are required.[201] This is especially crucial since *S. aureus* colonisation and infection is responsible for the majority of bacteria-associated SSTIs,[13] and an increased risk of SSTI recurrence, which impose a significant strain on healthcare resources.[11]

Current measures to break the cycle of recurrent infection include the disruption of *S. aureus* colonisation via administration of topical antimicrobials at various anatomic sites such as the nostrils to reduce nasal carriage.[11] The successful decolonisation of *S. aureus* however, has been hampered by the development of antimicrobial resistant strains over time, which subsequently makes it more difficult break the recurrent cycle of SSTIs.[11] Hence, novel treatment approaches are required.

If statins do serve as such novel agents, they should confer beneficial effects such as a reduced risk of SSTIs and/or a more rapid recovery from SSTIs for statin users compared to non-statin users. However, statins have also been associated with new-onset diabetes,[129] a risk factor for *S. aureus*-related SSTIs, which predisposes to recurrent SSTIs,[11, 157] potentially attenuating any plausible SSTI benefits that might be demonstrated by statins.

As such, the research reported in this chapter comprised of two separate analyses. A matched case-control study design was utilised in the primary analysis to evaluate the direct association between statin use and the risk of SSTIs. A secondary analysis was conducted to study the association between statin users who experienced SSTIs and the risk of diabetes. This chapter contains data which as yet, has not been submitted for publication.

5.1.1 Objectives

This study sought to determine if statin use conferred beneficial effects such as a reduced risk of SSTIs and/or a more rapid recovery from SSTIs amongst patients hospitalised due to an SSTI. The primary analysis of this study aimed to examine: (i) the association between statin use and the risk of SSTIs and (ii) if the use of statins was associated with improved clinical outcome indicators such as length of hospital stay and duration of discharge antibiotics prescribed.

Additionally, a secondary analysis was conducted within the SSTI cases only subgroup to determine if associations existed between statin use and: (i) the incidence of diabetes and (ii) clinical outcome indicators.

5.1.2 Potential significance of the research

Positive results from the primary analysis would potentially support a role for statins as viable novel therapeutic agents in the management of SSTIs, either through reducing the risk of severe SSTIs and/or facilitating a more rapid recovery from SSTIs.

Results from the secondary analysis determines the association between statin use amongst patients with SSTIs and diabetes, which could potentially identify whether statins attenuate or contribute to diabetes, an important risk factor of SSTIs.

5.2 Methods

5.2.1 Study design

A retrospective matched case-control study as outlined in Figure 5-1 was conducted on patients who were admitted as inpatients to the Medical Ward of Rockingham General Hospital, Western Australia, which is a public secondary hospital with slightly over 200 beds.

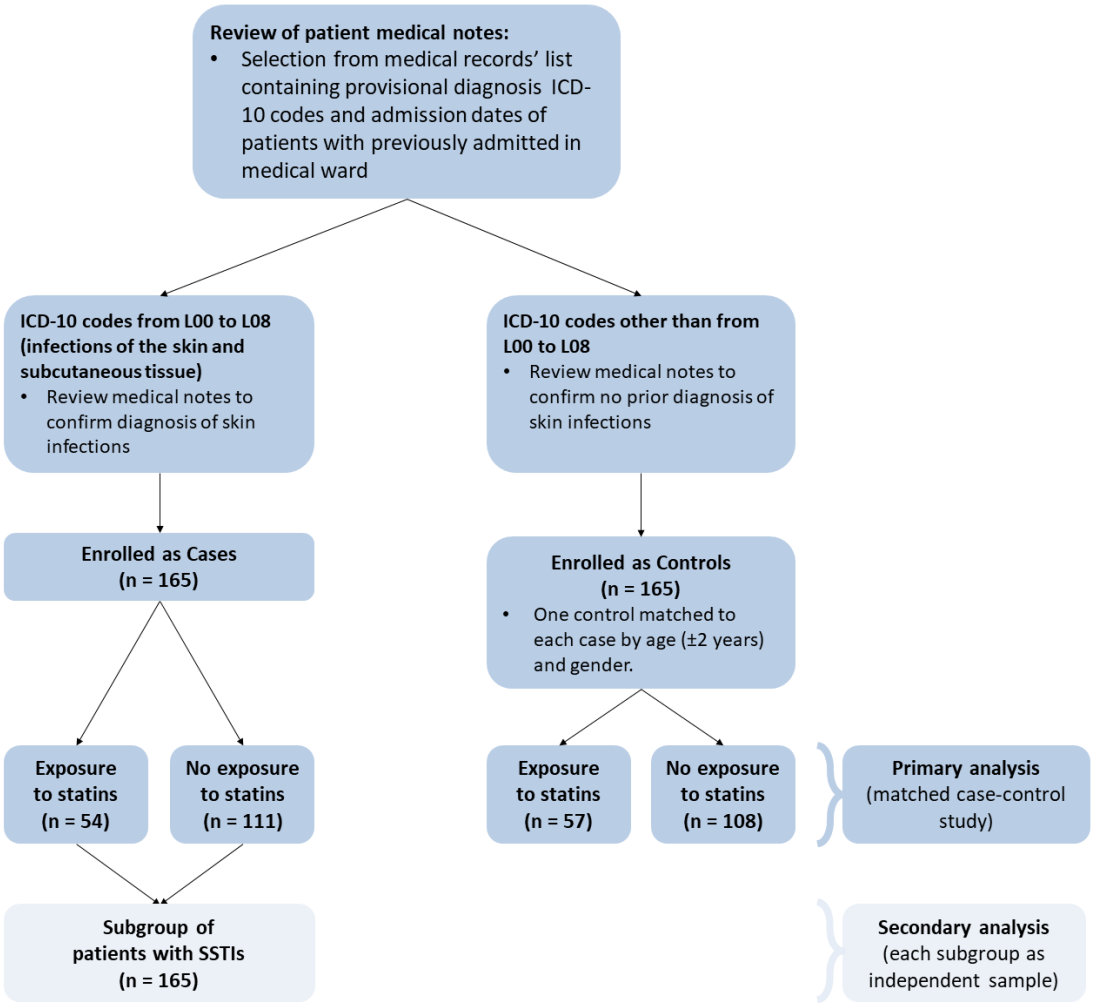

Figure 5-1: Diagram outlining methodology of study.
The procedure of enrolling cases and controls is shown, along with the population group(s) which the primary and secondary analyses were performed on. ICD-10, International Statistical Classification of Diseases and Related Health Problems - 10th revision.

5.2.1.1 Identification of cases and controls

Utilising a list containing provisional diagnosis according to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) and admission dates of patients admitted in the medical ward between January 2002 and January 2018, patients with ICD-10 codes from L00 to L08 (infections of the skin and subcutaneous tissue) were identified as potential cases. Although the list streamlined the process for the search of SSTI cases, coding of cases may sometimes be inaccurate. As such, the medical notes of these potential cases were reviewed by the primary investigator and thereafter, patients with

confirmed diagnosis of skin infections were enrolled as cases. Since the use of statins is recommended for adults aged 40 to 75 years to prevent cardiovascular diseases,[202] only patients who were 40 years and older admitted to the Medical Ward for SSTIs were selected as cases.

Patients with ICD-10 codes other than from L00 to L08 were marked as a pool of potential random controls. Since spurious associations due to confounders may arise from random sampling of controls, matching of cases to controls is performed to minimise this problem.[203] From the pool of controls, one potential control was matched to one selected case by age (±2 years) and gender. The potential control was confirmed as an enrolled control if the medical notes confirmed no prior diagnosis of SSTIs upon admission. A list of the various admission diagnoses for the confirmed controls have been included in Appendix 12. In situations where suitably matched controls admitted on the same day as cases could not be found, historic controls with other admission dates were utilised.

Cases of SSTIs which required surgical intervention were transferred to a tertiary hospital and not included in the study. Patients whose medical records were not available for their entire hospital stay were also excluded from this study.

5.2.1.2 Primary and secondary analyses

The primary analysis of this study aimed to determine: (i) the association between statin use and the risk of SSTIs and (ii) whether the use of statins was associated with improved clinical outcomes. Examining data from patients with SSTIs against the matched controls, if statins conferred beneficial effects against SSTIs, a statistically significant odds ratio (OR) of less than unity would be expected. In addition, outcome indicators such as length of hospital stay and duration of discharge antibiotics were evaluated to determine if statin use was associated with better outcomes. For example, a longer mean length of hospital stay and/or discharge antibiotics lasting longer than the upper limit of typical treatment for SSTIs (i.e. 14 days) would suggest poorer outcomes.

For the secondary analysis, data within the case subgroup (only SSTI cases) were examined as an independent small sample as the data were unmatched. The aims

were to determine the association between statin use and: (i) diabetes in SSTI cases and (ii) clinical outcome indicators.

5.2.2 Data collection

A customised data collection form was devised to collect relevant information (Appendix 13). Baseline demographics such as age and gender of each patient were recorded with a de-identified patient number for matching purposes. The age of the patients was further categorised as < 65 years and ≥ 65 years to determine if there was any difference in the prevalence of elderly patients (≥ 65 years) between the case and control groups. Upon admission, the Charlson Comorbidity Index, comorbidities which may influence SSTI risk (asthma, cancer, cirrhosis, chronic obstructive pulmonary disease [COPD], connective tissue disease, diabetes, human immunodeficiency virus [HIV] infection, obesity, and smoking status), and concurrent exposure of drugs commonly co-prescribed with statins (antiplatelets, ACEIs or ARBs, and beta blockers) were noted. The length of hospital stay and duration of antibiotics prescribed upon discharge were appraised as outcome indicators.

The Charlson Comorbidity Index contains 19 categories of comorbidities, each with an assigned weighted index, designed to reflect the cumulative probability of 1 year mortality.[204] This index has been shown to be a good predictor of mortality in patients with *S. aureus* bacteraemia,[205] and has been used to control comorbidities in studies investigating risk factors for death due to bacteremia.[155, 205] A higher score is indicative of a more severe comorbidity burden. In this study, the index was calculated for each patient upon admission as a baseline reference of comorbidity severity for comparison between both case and control patients.

Comorbidities may contribute to SSTI risk and severity as intrinsic risk factors or due to immunosuppression.[13] Diabetes and obesity are not only risk factors for SSTIs,[13] but they are also risk factors for impaired wound healing and wound complications, as is cigarette smoking.[206] Patients with cancer, cirrhosis, and HIV infection are immunocompromised and thus susceptible to SSTIs.[13] Patients with asthma or COPD may be susceptible to bacterial infections due to regular long term inhaled corticosteroids with occasional oral immunosuppressive corticosteroids for

exacerbation,[207] while patients with connective tissue diseases such as systemic lupus erythematosus and rheumatoid arthritis are associated with chronic immunosuppressive treatment.[208] As such, asthma, cancer, cirrhosis, COPD, connective tissue disease, diabetes, HIV infection, obesity, and cigarette smoking status were included as confounding factors in the primary analysis.

Statins are commonly prescribed together with aspirin (an antiplatelet), ACEIs or ARBs, and beta blockers for primary and secondary prevention of cardiovascular diseases.[209, 210] Thus upon admission, the use of statins, antiplatelets, ACEIs or ARBs, and beta blockers were factored into the primary analysis.

Patients were classified as statin users if they were found to be on statins for at least three months immediately prior to admission, as determined by medication records. Non-statin users were defined as patients with no history of statin use within three months immediately prior to admission. Users and non-users of antiplatelets, ACEIs or ARBs, and beta blockers were similarly determined.

5.2.3 Sample size calculations and statistical analysis

Assuming a statin exposure of 40% in controls,[211] in order to detect with 80% power a protective effect of OR of 0.5 with 95% CI and 1:1 ratio of cases to controls, it was determined that at least 152 cases and 152 controls (total sample size of 304 patients) would be required.[212]

Demographic characteristics with continuous variables (age, Charlson Comorbidity Index, and length of stay) were tested with the Shapiro-Wilk's test for normality. If the data were normally distributed, the two-sample t-test was utilised. Otherwise, the nonparametric Mann-Whitney U test was performed. Differences in categorical characteristics (gender, age groups, Charlson Comorbidity Index groups, comorbidities on admission, class of concurrent drug exposure on admission, and grouped duration of antibiotics on discharge) were determined by the Chi-square test.

Since conditional logistic regression minimises sparse data bias and has become a standard for analysing matched case–control data,[203] the method was employed with SSTI as the outcome in the primary analysis to determine if there was any significant

associations with variables such as statin use, by estimating the OR and 95% CI.[213] Matched by age (±2 years) and gender, comorbidities (asthma, cancer, cirrhosis, COPD, connective tissue disease, diabetes, HIV infection, obesity, and cigarette smoking status) and drug exposure to statins, antiplatelets, ACEIs or ARBs, and beta blockers on admission were used as covariates in the regression model. To detect significant relationships within statin users and non-statin users paired with clinical outcomes (length of hospital stay or duration of discharge antibiotics), the Fisher's exact test (two-sided) was reported together with the OR and 95% CI.

Due to the relatively small sample size for the secondary analysis (case subgroup with only SSTI patients), variables were stratified into a 2 x 2 contingency table and the Fisher's exact test (two-sided) was reported along with the relative risk (RR) and 95% CI. This helped indicate significant relationships within statin users and non-statin users paired with risk factors (diabetes status) and outcome indicators (length of stay or duration of discharge antibiotics) in patients with SSTIs.

Statistical analyses were performed using SPSS Statistics Version 25.0 (IBM Corp, Armonk, NY), with statistically significant associations defined as $p < 0.05$.

5.2.4 Ethics approval

This study was approved by the South Metropolitan Area Health Service, Western Australia (12/285, Appendix 14), and reciprocal ethics approval was granted by Curtin University, Western Australia (HR155/2015, Appendix 15)

5.3 Results

5.3.1 Baseline demographics

The baseline demographics are presented in Table 5-1. A total of 330 patients comprising 165 cases of SSTIs matched with 165 controls by age (± 2 years) and gender were included in this study. Both groups had similar baseline parameters with no significant differences in terms of Charlson Comorbidity Index, length of stay upon discharge, comorbidities on admission (asthma, cancer, cirrhosis, COPD, connective tissue disease, diabetes, HIV infection, obesity, and cigarette smoking status), and concurrent drug exposures of statins, antiplatelets, ACEIs or ARBs, and

beta blockers (Table 5-1; Chi-square test, $p > 0.051$). However, the groups differed significantly in terms of obesity status ($p < 0.001$), which was factored in the conditional logistic regression analysis.

Table 5-1: Demographics of 165 cases (patients with SSTIs) matched with 165 controls (patients without SSTIs). [†]

Variable	Cases (%) *n = 165*	Controls (%) *n = 165*	p-value
Gender			
Male	86 (52.1)	86 (52.1)	1.000
Female	79 (47.9)	79 (47.9)	
Age, years			
Mean ± SD	63.48 ± 14.06	63.58 ± 14.02	0.946
< 65 years	84 (50.9)	85 (51.5)	0.912
≥ 65 years	81 (49.1)	80 (48.5)	
Charlson Comorbidity Index			
Mean ± SD	3.76 ± 2.95	3.78 ± 2.71	0.638
≤ 3	91 (55.2)	81 (49.1)	0.270
> 3	74 (44.8)	84 (50.9)	
Outcome Indicator			
Length of stay, days			
Mean ± SD	6.11 ± 11.28	6.10 ± 10.58	0.415
Comorbidities on admission			
Asthma			
Yes	25 (15.2)	24 (14.5)	0.877
No	140 (84.8)	141 (85.5)	
Cancer			
Yes	18 (10.9)	19 (11.5)	0.861
No	147 (89.1)	146 (88.5)	
Cirrhosis			
Yes	3 (1.8)	1 (0.6)	0.314
No	162 (98.2)	164 (99.4)	
COPD			
Yes	16 (9.7)	17 (10.3)	0.854
No	149 (90.3)	148 (89.7)	
Connective tissue diseases			
Yes	10 (6.1)	6 (3.6)	0.305
No	155 (93.9)	159 (96.4)	
Diabetes			
Yes	38 (23)	39 (23.6)	0.896
No	127 (77)	126 (76.4)	
HIV infection			
Yes	1 (0.6%)	0 (0)	Nil positive cases in control group
No	164 (99.4%)	165 (100)	
Obesity			
Yes	52 (31.5)	24 (14.5)	**< 0.001**
No	113 (68.5)	141 (85.5)	
Smoker (current)			
Yes	21 (12.7)	23 (13.9)	0.746
No	144 (87.3)	142 (86.1)	
Concurrent drug exposure on admission			
Statins			
None	111 (67.3)	108 (65.5)	0.649
Atorvastatin	28 (17)	28 (17)	
Pravastatin	6 (3.6)	4 (2.4)	
Rosuvastatin	10 (6.1)	17 (10.3)	
Simvastatin	10 (6.1)	8 (4.8)	
Antiplatelets			
Non-user	121 (73.3)	116 (70.3)	0.541
User	44 (26.7)	49 (29.7)	
ACEIs or ARBs			
Non-user	105 (63.6)	110 (66.7)	0.564
User	60 (36.4)	55 (33.3)	
Beta blockers			
Non-user	137 (83)	135 (81.8)	0.772
User	28 (17)	30 (18.2)	

([†]) Mann-Whitney U test was performed on continuous variables (age, Charlson Comorbidity Index, and length of stay). Chi-square test was performed on categorical characteristics (gender, age groups, Charlson Comorbidity Index groups, comorbidities on admission, and class of concurrent drug exposure). COPD, Chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; SD, standard deviation.

5.3.2 Primary analysis

5.3.2.1 Statin use and the risk of SSTIs

It was found that only obesity status was significantly associated with an increased risk of SSTIs in this study (Table 5-2; OR = 2.968; 95% CI = [1.609 – 5.476]; p < 0.001). The use of ATV, PRV, RSV, and SMV was not significantly associated with SSTIs (Table 5-2; p > 0.05). The other variables of comorbidities (asthma, cancer, cirrhosis, COPD, diabetes, and smoking statuses) or concurrent drug exposures (antiplatelets, ACEIs or ARBs, and beta blockers) were also not significantly associated with a risk of SSTIs (Table 5-2; p > 0.05). HIV infection status was omitted as it could not be calculated due to absence of this comorbidity in the control group.

Table 5-2: Primary analysis (i) association between statin use and risk of SSTIs (n = 330).[†]

Variable	165 cases against 165 controls		
	Odds ratio[#]	**95% CI**[#]	**p-value**
Comorbidities			
Asthma			
Cancer	0.850	0.391 – 1.844	0.680
Cirrhosis	2.873	0.244 – 33.810	0.401
COPD	0.746	0.335 – 1.660	0.473
Connective tissue disease	1.554	0.407 – 5.938	0.519
Diabetes	0.860	0.456 – 1.619	0.640
Obesity	2.968	1.609 – 5.476	**< 0.001**
Smoker (current)	1.097	0.526 – 2.288	0.805
Drug exposure			
Non-statin user	1	Reference	
Atorvastatin	1.195	0.566 – 2.526	0.640
Pravastatin	1.756	0.444 – 6.946	0.422
Rosuvastatin	0.528	0.202 – 1.380	0.192
Simvastatin	1.353	0.471 – 3.888	0.574
Antiplatelet users	0.846	0.470 – 1.523	0.577
ACEI or ARB users	1.206	0.694 – 2.093	0.507
Beta blocker users	0.791	0.389 – 1.609	0.518

([†]) Conditional logistic regression was applied due to the matching of cases to controls by age (± 2 years) and gender. ([#]) Odds ratio and 95% CI matched for age and gender, and adjusted for comorbidities (asthma, cancer, cirrhosis, COPD, connective tissue disease, diabetes, obesity, and smoking status) and drug exposure (statins, antiplatelets, ACEIs or ARBs, and beta blockers) on admission. HIV infection status was omitted due to absence of this comorbidity in the control group. ACEI, angiotensin-converting enzyme inhibitor; ARB, Angiotensin II receptor blocker; COPD, Chronic obstructive pulmonary disease; HIV, human immunodeficiency virus.

5.3.2.2 Statin use and clinical outcomes in total sample population

Compared to non-statin users, statin users were not associated with any significant improvements in clinical outcomes as shown in Table 5-3. The mean length of hospital stay (six days) and typical antibiotic treatment duration for SSTIs (i.e. 14 days) were not statistically significant between statin users and non-statin users (Table 5-3; $p > 0.05$).

Table 5-3: Primary analysis (ii) association between statin use and clinical outcome indicators in total sample population (n = 330).[‡]

Variable	Statin users (%) *n = 111*	Non-statin users (%) *n =219*	Odds ratio[#] (95% CI)	p-value
Clinical outcome indicators				
Length of stay				
≤ 6 days	82 (73.9)	161 (73.5)	0.982	1.000
> 6 days	29 (26.1)	58 (26.5)	(0.584 – 1.650)	
Duration of discharge antibiotics				
≤ 14 days	104 (93.7)	200 (91.3)	0.709	0.522
> 14 days	7 (6.3)	19 (8.7)	(0.289 – 1.740)	

([‡]) Variables were stratified into a 2 x 2 contingency table and two-sided Fisher's exact test was conducted for each variable. ([#]) Odds ratio was calculated due to samples being taken from a matched case-control study design.

5.3.3 Secondary analysis

5.3.3.1 Statin use and diabetes in SSTI cases only

Within the unmatched subgroup of SSTI cases only, obesity (Table 5-4; RR = 2.173; 95% CI = [1.261 – 3.746]; p = 0.009) and ATV (Table 5-4; RR = 2.854; 95% CI = [1.699 – 4.795]; p = 0.001) were significantly associated with an increased risk of diabetes.

Table 5-4: Secondary analysis (i) association between statin use and diabetes in SSTI cases only (n = 165).[‡]

Variable	Diabetics (%) *n = 38*	Non-diabetics (%) *n = 127*	Relative risk[#] (95% CI)	p-value
Comorbidities on admission				
Asthma				
Yes	8 (21.1)	17 (13.4)	1.493	0.302
No	30 (78.9)	110 (86.6)	(0.777 – 2.871)	
Cancer				
Yes	5 (13.2)	13 (10.2)	1.237	0.566
No	33 (86.8)	114 (89.8)	(0.554 – 2.763)	
Cirrhosis				
Yes	1 (2.6)	2 (1.6)	1.459	0.547
No	37 (97.4)	125 (98.4)	(0.287 – 7.413)	
COPD				
Yes	5 (13.2)	11 (8.7)	1.411	0.531
No	33 (86.8)	116 (91.3)	(0.643 – 3.099)	
Connective tissue diseases				
Yes	0 (0)	10 (7.9)	Nil positive cases in diabetic group	Nil positive cases in diabetic group
No	38 (100)	117 (92.1)		
HIV infection				
Yes	0 (0)	1 (0.8)	Nil positive cases in diabetic group	Nil positive cases in diabetic group
No	38 (100)	126 (99.2)		
Obesity				
Yes	19 (50)	33 (26)	2.173	**0.009**
No	19 (50)	94 (74)	(1.261 – 3.746)	
Smoker (current)				
Yes	6 (15.8)	15 (11.8)	1.286	0.580
No	32 (84.2)	112 (88.2)	(0.612 – 2.700)	
Drug exposure on admission				
Statins				
Atorvastatin users	14 (36.8)	14 (11)	2.854	**0.001**
Non-atorvastatin users	24 (63.2)	113 (89)	(1.699 – 4.795)	
Pravastatin users	1 (2.6)	5 (3.9)	0.716	1.000
Non-pravastatin users	37 (97.4)	122 (96.1)	(0.117 – 4.382)	
Rosuvastatin	2 (5.3)	8 (6.3)	0.861	1.000
Non-rosuvastatin users	36 (94.7)	119 (93.7)	(0.241 – 3.073)	
Simvastatin users	3 (7.9)	7 (5.5)	1.329	0.698
Non-simvastatin users	35 (92.1)	120 (94.5)	(0.493 – 3.578)	

([‡]) Due to the small sample size, variables were stratified into a 2 x 2 contingency table and two-sided Fisher's exact test was conducted. ([#]) Relative risk was calculated due to samples being taken from an independent sample.

5.3.3.2 Statin use and clinical outcomes in SSTI cases only

Within the group of SSTI cases only, the mean length of hospital stay (six days) and typical antibiotic treatment duration for SSTIs (i.e. 14 days) were not statistically significant between statin users and non-statin users (Table 5-5; $p > 0.05$).

Table 5-5: Secondary analysis (ii) association between statin use and clinical outcome indicators in SSTI cases only (n = 165).[‡]

Variable	Statin users (%) *n = 54*	Non-statin users (%) *n =111*	Relative risk[#] (95% CI)	p-value
Outcome Indicators				
Length of stay				
≤ 6 days	43 (79.6)	75 (67.6)	0.642	0.141
> 6 days	11 (20.4)	36 (32.4)	(0.363 – 1.135)	
Duration of discharge antibiotics				
≤ 14 days	48 (88.9)	94 (84.7)	0.772	0.633
> 14 days	6 (11.1)	17 (15.3)	(0.374 – 1.594)	

([‡]) Due to the relatively small sample size, variables were stratified into a 2 x 2 contingency table and two-sided Fisher's exact test was conducted. ([#]) Relative risk was calculated due to samples being taken from an independent sample.

5.3.4 Summary of results

The pertinent results of the primary and secondary analyses have been summarised in Figure 5-2 to facilitate the discussion that follows.

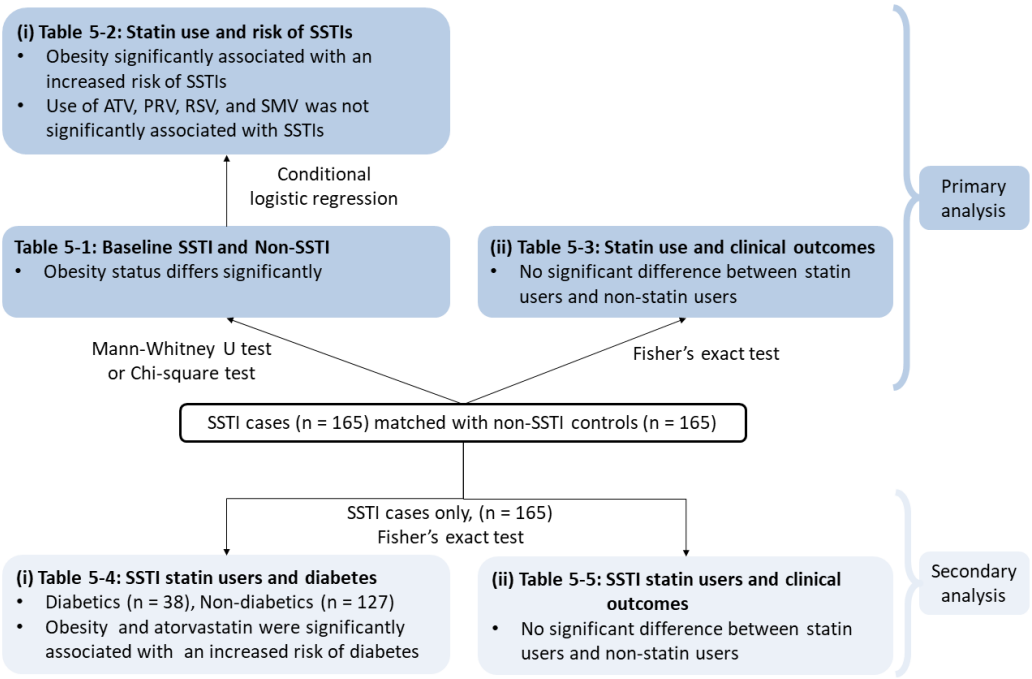

Figure 5-2: Flowchart summarising pertinent results of the primary and secondary analyses with relevant table references in bold.

5.4 Discussion

5.4.1 Statin use and direct risk of SSTIs

In the primary analysis, the use of ATV, PRV, RSV, and SMV was not significantly associated with SSTIs (Table 5-2; $p > 0.05$). When compared to non-statin users, statin users did not demonstrate any significant benefits in clinical outcomes such as the mean length of hospital stay or typical duration of antibiotic treatment (Table 5-3; $p > 0.05$).

Although there have been reviews which concluded that statins had potential to protect against infections,[96, 214, 215] conflicting data from other reviews also exist.[98, 171, 216] There is a possibility of publication bias, whereby studies which demonstrate favourable effects of statins in infections were selected for publication over studies which showed neutral or even adverse statin effects of statins in patients with infections.[97, 216] The evidence in this study aligned with the latter group which does not corroborate the hypothesis that statins exert beneficial effects on infections, specifically SSTIs.

It was noted that many of the positive reports of statins' favourable effects against infections were observational studies which could be subjected to the "healthy user effect".[159, 171] The healthy user effect refers to selective bias whereby motivated patients exhibit health-seeking traits such as consuming healthy diets and exercising regularly.[162] This study was unable to evaluate the influence of the healthy user effect, elaborated later as a study limitation (Section 5.4.3).

5.4.2 Statin use and risk factors for SSTIs

Obesity status was significantly greater in the SSTI cases compared to the controls at baseline (Table 5-1). It was still found to be a significant risk factor for SSTIs after adjustment in the regression analysis (Table 5-2), and is also significantly associated with diabetes in SSTI patients (Table 5-4). These results would be anticipated because in addition to obesity and diabetes both being risk factors for SSTIs,[13] obesity is also a risk factor for diabetes.[217] The other comorbities on admission (asthma, cancer, cirrhosis, COPD, and connective tissue diseases) were not shown to be significant risk factors for SSTIs (Table 5-2).

The significant association of obesity status and SSTIs shown in Table 5-2 aligned with a study which showed a strong association between obesity and SSTIs among men, confirmed with an increased risk of filled prescriptions for antibiotics specifically prescribed for SSTIs related to *S. aureus* (dicloxacillin and flucloxacillin).[218]

Langley et al. demonstrated that both obesity and diabetes were indeed found to be important risk factors for SSTIs.[219] This was likely because obese patients have impaired immune systems, skin barrier functions, and/or lung physiology, while patients with diabetes might be immunocompromised and have poor wound healing abilities.[219]

Statins might reasonably be expected to benefit obese patients.[220] The use of statins such as ATV, RSV, and SMV confer favourable lipid modifications in the management of obesity such as increasing the levels of high-density lipoprotein cholesterol and reduction of LDL-C and triglycerides.[221] Although such desired lipid profiles are recommended in guidelines for obesity management,[222] statins have not been specifically recommended in the pharmacological management of obesity.[222-224]

On the contrary, statins have been associated with increased risk of obesity as well as diabetes.[225] Statin users, compared to non-statin users, were more likely to be sedentary and less participative in moderate exercise,[225] and the body mass index of statin users increased at a faster rate.[226] It was hypothesised that statins were associated with obesity because patients started statins were under the impression that they did not have to restrict their current diet.[226] However, the use of statins has been also associated with weight gain in mice,[121] whereby the human psychological factor bears no influence. Further research could be performed to verify the association between statins and obesity in humans. In this study however, no significant association between statins and obesity status was found in patients with SSTIs (Supplementary data, Appendix 16-1).

Although there were no direct significant associations detected between statin use and SSTI risk in the primary analysis (Table 5-2), ATV was associated with an

increased RR of diabetes in the subgroup of SSTI patients (Table 5-4; RR = 2.854; CI = [1.699 – 4.795]; $p < 0.001$). The increased risk of diabetes associated with ATV was in alignment with the results from Chapter Four and several other studies.[129, 227, 228] In the subgroup of controls without SSTIs, RSV was associated with a significant increased RR of diabetes as well (Supplementary data, Appendix 16-2). Since the risk of diabetes predisposes patients to *S. aureus*-related SSTIs, which in turn increases the probability of recurrent SSTIs,[11, 157] the results suggest ATV is unlikely to mitigate diabetes to disrupt recurrent SSTIs, but rather, be associated with an increased risk of diabetes-induced recurrent SSTIs instead.

Having found that statins confer no beneficial impact on direct SSTI risk but rather, positive associations with SSTI risk factors diabetes and possibly obesity, it would appear that statins are unlikely to either serve as novel therapeutic agents for SSTIs or curb the recurrent SSTI cycle.

5.4.3 Limitations

Despite efforts to identify and adjust for known confounding factors (Section 5.2.2), there may be other confounders which influenced the results of this retrospective case-control study, which was performed on a relatively small sample size of a total of 330 patients. This was slightly in excess of the calculated minimum sample size of 304 patients to detect the protective effect of statins (Section 5.2.3). A larger sample size and matching of one case to more than one control might present more significant results of interest.

The healthy user effect corresponds closely with socio-economic status.[162] Although the residential electorate is reflective of patients' socio-economic status,[168] it could not be used in this study as a surrogate indicator of the healthy user effect because the data here would be biased towards the hospital district and its vicinity, where most of the patients lived.

Due to the retrospective data for this study being collected over more than 10 years (2002 to 2018), there is a possibility of practice changes influencing the study outcomes. As such, the Charlson Comorbidity Index was utilised in the analysis at baseline. Since there were no significant differences between the cases and controls

(Table 5-1), we could be reasonably assured that despite possible practice changes over time, the cases and controls did not differ significantly in terms of comorbidities at baseline. Moreover, the empirical treatment guidelines for hospitalised patients with severe skin infections such as cellulitis has remained largely unchanged between 2003 to 2019 according to the Australian Therapeutic Guidelines for antibiotics.[229, 230] For example, suspected *S. aureus* related infections are treated with intravenous flucloxacillin, patients with non-severe penicillin hypersensitivity are treated with intravenous cefazolin, and patients with severe penicillin hypersensitivity are treated with intravenous vancomycin.[229, 230]

5.5 Conclusions

Obesity status was found to be a significant risk factor for SSTIs. The use of statins (ATV, PRV, RSV, and SMV) was not significantly associated with SSTIs and statin users did not demonstrate better clinical outcomes compared to non-statin users.. However, ATV was significantly associated with diabetes in patients with SSTIs, which suggests ATV is more likely to contribute to the recurrence of SSTIs via association with diabetes as a risk factor for *S. aureus*-related SSTIs, which predisposes to recurrent SSTIs. There was no significant difference in clinical outcomes between statin users and non-statin users in the subpopulation of patients with SSTIs.

The hypothesis of using statins (ATV, PRV, RSV, and SMV) as novel therapeutic agents appeared unlikely from this study. However, the clinical effects of two other statin members, LVS and PTV, have not been studied due to their unregistered status in Australia. The next chapter reconciles all the accumulated evidence and evaluates the likelihood of statins (including LVS and PTV) serving as a potential novel antibacterial agent against SSTIs.

CHAPTER SIX

6. Discussion of Accumulated Evidence

6.1 Overview

Having accumulated laboratory evidence (Chapter Three) and clinical evidence (Chapters Four and Five), the associations of statins and bacterial SSTIs are evaluated to determine if basic scientific research (laboratory evidence) translated to viable applied research (clinical evidence).

Based on the reconciled evidence and reflections on the strengths and limitations of this study, suggestions for future research are recommended.

6.2 Reconciliation of laboratory evidence with clinical evidence

6.2.1 Most suitable statin(s) as novel adjuvant(s)/treatment(s) for SSTIs

Drugs suitable as novel therapeutic agents for SSTIs may be identified when the antibacterial activity exhibited in the laboratory is complemented with beneficial clinical effects on SSTIs. The lack of evidence from either fields of research would mitigate the overall support for the hypothesis of statins' potential as novel therapeutic agents, or even invalidate the hypothesis if evidence from one field contradicts that of the other.

The ideal novel statin adjuvant/treatment for SSTIs should thus have a combination of: (i) potent antibacterial activity (low MICs) against a wide spectrum of bacterial pathogens causing SSTIs, especially strains which are drug resistant and/or cause complicated SSTIs (Chapter Three), (ii) the ability to reduce SSTI risk directly (Chapters Four and Five) and improve clinical outcomes in patients with SSTIs (Chapter Five), along with (iii) beneficial or neutral effects on the risk of diabetes and obesity, since diabetes is a risk factor for *S. aureus*-related SSTIs, which predisposes to recurrent SSTIs (Chapters Four and Five), and obesity is also a significant risk factor of SSTIs (Chapter Five).

Using a translational framework, the path of each statin's likelihood in realising the potential as a novel adjuvant/treatment for SSTIs will be assessed by reconciling positive *in vitro* antibacterial activity with beneficial *in vivo* effects on the direct risk

of SSTIs and risk factors of SSTIs such as diabetes. By the process of elimination, the most likely statin(s) suitable as novel adjuvants/treatments will emerge.

6.2.1.1 Likelihood of ATV, FLV, PTV, and SMV

Out of the seven parent statins (ATV, FLV, LVS, PTV, PRV, RSV, and SMV) and three selected statin metabolites (LVS-OH acid, PTV-lactone, and SMV-OH acid) tested, SMV (MIC = 64 μg/mL) and PTV-lactone (MIC = 128 μg/mL) exerted the greatest antibacterial activity against MSSA, followed by ATV and FLV (both MIC = 256 μg/mL; Figure 3-2a).

Spectrophotometric analysis revealed that SMV-OH acid, PTV, and LVS-OH exerted potential antibacterial activity against MSSA, which could not be discerned by the unaided eye (Figure 3-2c). Furthermore, SMV-OH acid might be active against *E. coli* (Figure 3-3) and *S. marcescens* (Figure 3-4) at higher drug concentrations (> 256 μg/mL).

As such, the clinical effects of SMV and PTV on SSTIs would be of greatest interest in identifying the most suitable statin as a novel adjuvant/treatment for SSTIs, because both the respective parent drug and metabolite demonstrated at least some *in vitro* antibacterial activity detectable by spectrophotometry. The clinical effects of ATV and FLV, which exhibited lower MICs compared to SMV or PTV-lactone, would also be of considerable interest.

However, since none of the statins/metabolites exerted *in vitro* antibacterial activity (at drug concentrations ≤ 256 μg/mL) against *E. coli* (Figure 3-3), *S. marcescens* (Figure 3-4), or *P. aeruginosa* (Figure 3-5) which may cause complicated SSTIs, any beneficial clinical effect observed will be relevant only to MSSA-related SSTIs.

Since PTV is currently not registered for clinical use in Australia,[231] its association with SSTI risk could not be assessed. However, whilst different clinical studies reported conflicting effects of statins (ATV, FLV, PRV, RSV, and SMV) on total adiponectin levels, it was only PTV that consistently demonstrated significant increases in total adiponectin levels.[232]

Adiponectin is an adipokine (cytokine secreted by adipose tissues) which modulates the human innate immune system to confer protective effects against inflammation and insulin resistance,[233] as well as regulate keratinocyte proliferation to improve wound healing.[234] It has been proposed that by increasing adiponectin levels or activation of adiponectin receptors, the risk of diabetes and obesity may be reduced.[235, 236] Since *in vitro* antibacterial activity has been demonstrated by PTV-lactone along with potential activity by PTV (Figure 3-2), and both diabetes and obesity are significant risk factors of SSTIs (Section 5.4.2), it is plausible that PTV-lactone (and perhaps PTV) could serve as novel adjuvant(s)/treatment(s) for SSTIs via intrinsic antibacterial activity and *in vivo* increase of adiponectin levels, which in turn reduces risk of diabetes and obesity. Further clinical research in a country which has PTV registered for clinical use would help corroborate the viability of PTV-lactone and/or PTV as a novel therapeutic agent for SSTIs.

Of all the statins tested, SMV exhibited the greatest *in vitro* antibacterial activity against MSSA in this study. Several other studies have demonstrated its activity against various other bacterial strains, including MRSA, *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, albeit higher MICs were required for the Gram-negative bacteria (Tables 2-1 and 2-2). Based on outpatient evidence, SMV was associated with significant increases in risk of both SSTIs (at 91 days; Figure 4-3) and diabetes (at 91 days; Figure 4-4). There were no significant associations of SMV with the risk of SSTIs or diabetes from the inpatient data (Tables 5-2 and 5-4 respectively), albeit reviews of other clinical studies have associated SMV use with significant risks of diabetes.[129, 237] SMV-OH acid is an active metabolite of SMV,[78] hence its clinical effects would be akin to that of the parent statin. As such, despite SMV exhibiting antibacterial effects and SMV-OH acid demonstrating a potential antibacterial effect, significant increased clinical risks (SSTIs and diabetes) likely outweighs any benefit from SMV as a novel therapeutic agent for SSTIs.

In this study, ATV exhibited significant *in vitro* antibacterial activity against MSSA, whilst other researchers have shown its activity against various other bacterial strains such as MRSA, *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* (Tables 2-1 and 2-2). However, ATV was associated with significant increases in risk of SSTIs (at 91 days; Figure 4-3) and diabetes (at 91 days; Figure 4-4), together with increased

risk of diabetes in patients with SSTIs (Table 5-4). Any benefits from ATV as a novel therapy therefore would unlikely prevail over the combined risks of SSTIs and diabetes associated with ATV use.

Although FLV demonstrated *in vitro* antibacterial activity against MSSA and was not significantly associated with risks of SSTIs or diabetes at 91 days in this study (Figures 4-3 and 4-4), it has been associated with significant diabetes risk in other studies.[237] The non-significant results at 91 days was likely due to the low use of FLV in Australia, as shown by the relatively small sample size of outpatient claims by veterans and their dependents across the nation (Figure 4-3 and 4-4) and no hospitalised patients using FLV in the inpatient data (Table 5-1). In view of the small sample size, yet being associated with increased SSTI risk at 365 days (Figure 4-3), being associated with significant diabetes risk in other studies,[237] and having a relatively high MIC (256 μg/mL; Figure 3-2a) against MSSA, the clinical risks are still likely greater than potential benefits from use of FLV as a novel therapeutic agent for SSTIs.

6.2.1.2 Likelihood of LVS, PRV, and RSV

The remaining three parent statins (LVS, PRV, and RSV) were found to have no antibacterial activity against MSSA (Figure 3-2d). Hence, unless they demonstrate exceptional clinical benefits in patients with SSTIs, they would be unlikely candidates as novel adjuvants/treatments for SSTIs. Both RSV and PRV were associated with significant SSTI risk over 365 days (Figure 4-3), and studies have associated both RSV and PRV with significant risk of diabetes.[237] The clinical effects of LVS could not be evaluated because like PTV, it is not registered for clinical use in Australia.[231] Unlike PTV however, LVS has been associated with diabetes risk.[129] Hence LVS, PRV, and RSV would unlikely serve as viable novel adjuvants/treatments for SSTIs.

6.3 Strengths and limitations of accumulated evidence

The limitations pertaining to the individual laboratory and clinical work include issues involving the insolubility of SMV (Section 3.4.4), the conditions required for SSA studies (Section 4.4.3), and shortcomings of the case-control study conducted

(Section 5.4.3). These have been acknowledged and addressed as appropriately as possible in the respective Sections of the thesis.

Having discerned the likelihood of each statin being a potential novel adjuvant/treatment for SSTIs in Section 6.2, the results are summarised in Table 6-1 to facilitate reflections on the strengths and limitations of the overarching research on the relationships between statins and bacterial SSTIs.

Table 6-1: Tabulated summary of evidence in a translational research framework to identify statin(s) suitable as novel adjuvant(s)/treatment(s) for SSTIs.

	Basic research ⟷		**Applied research**	➡ **Outcome**	
Statin	Laboratory evidence	Outpatient evidence		Inpatient evidence	Conclusion
	Activity against MSSA?	↑SSTI risk? (91 days)	↑diabetes risk? (91 days)	↑diabetes risk with SSTI?	Novel therapy for SSTI?
Parent drug					
ATV	Yes	Yes	Yes	Yes	Unlikely
FLV	Yes	ns[†]	ns	NT[‡]	Unlikely
LVS	No	NT	NT	NT	Unlikely
PTV	Potential	NT	NT	NT	Oral agent?
PRV	No	Yes	Yes	ns	Unlikely
RSV	No	Yes	ns	ns	Unlikely
SMV	Yes	Yes	Yes	ns	Unlikely
Metabolite					
LVS-OH acid	Potential	NT (parent)	NT (parent)	NT (parent)	Unlikely
PTV-lactone	Yes	NT (parent)	NT (parent)	NT (parent)	Topical agent?
SMV-OH acid	Potential	Yes (parent)	Yes (parent)	ns (parent)	Unlikely

([†]) Although risk of SSTI for FLV was not significant at 91 days, the risk was significant at 365 days. The non-significant result at 91 days was likely due to the small sample size. ([‡]) Not tested in the hospital setting as none of the sampled patients were taking FLV. The parent statins LVS and PTV are not registered for clinical use in Australia, hence the clinical effects of LVS, PTV, and their metabolites were not tested. Abbreviations: ns, not significant; NT, not tested.

6.3.1 Importance and limitations of clinical evidence

Increased risks associated with SSTIs and diabetes were the main reasons for statins being assessed as unlikely novel therapies for SSTIs. Despite theoretical benefits of drug repurposing (Section 1.3.4), the clinical evidence gathered from this study and other literature for statins registered for clinical use in Australia (ATV, FLV, PRV,

RSV, and SMV) revealed a small but significant increased risk of diabetes (Table 6-1), potentially predisposing patients to *S. aureus*-related SSTIs, which in turn raises the probability of recurrent SSTIs.[11, 157] Additionally, obesity has been identified as a significant risk factor for SSTIs (Table 5-2).

The SSA analysis in Chapter Four was instrumental in revealing that first-time statin users are at increased risk of SSTIs and this risk was likely independent of diabetes status or the healthy user effect (Section 4.4.2). Statins may increase the risk of SSTIs through a direct mechanism (plausibly via increase of Treg cells and inhibition of Th1 and Th17 cells, reducing innate immunity [Section 4.4.1.2]), or through an indirect mechanism (reduction of LDL-C and coenzyme Q10 levels within 91 days of statin commencement, which increases the risk of diabetes, in turn a risk factor for SSTIs [Section 4.4.1.1]).

Furthermore, the evaluation of risks at different time periods of 91, 182, and 365 days revealed the risk of SSTIs (Figure 4-3) and diabetes (Figure 4-4) occurred as soon as 91 days after statin commencement, particularly for ATV and SMV. The time period by which diabetes manifested after statins were started aligned with other studies which suggest reduced plasma levels of LDL-C and/or coenzyme Q10 being plausible mechanisms for the associated risk with diabetes. Hence there would be a need to increase awareness of this risk amongst physicians, who should monitor the blood glucose levels of statin users and advise accordingly.

Although the case-control study in Chapter Five could not corroborate that statins increased the risk of SSTIs significantly, it validated that obesity was a significant risk factor for SSTIs (Table 5-2), and that SSTI patients using ATV were associated with significantly increased risk of diabetes (Table 5-4), which predisposes them to *S. aureus*-related SSTIs and eventually, recurrent SSTIs.[11, 157] Literature suggests statins may increase the risk of obesity, but there was no significant association found between statins and obesity in this study (Section 5.4.2).

Clinical effects of LVS and PTV could not be studied due to their unregistered status in Australia. From available literature however, LVS has been associated with diabetes,[129] while PTV has been consistently associated with significant increases in

adiponectin levels, an effect suggested to exert beneficial effects on obesity and diabetes,[232] which would in turn mitigate SSTI risk factors. With such favourable systemic effects on adiponectin levels, there may be a possibility that oral PTV could potentially serve as a novel therapeutic agent for SSTIs. Since it has been found that the high-molecular-weight adiponectin isoforms are responsible for the favourable effects, it would benefit future studies on PTV's clinical effects to focus on the high-molecular-weight to total adiponectin ratios rather than just total adiponectin levels alone.[235] The association between PTV and diabetes is being further investigated by other researchers in a randomised controlled trial,[200] and their results would help verify if PTV exerts favourable effects on diabetes as demonstrated by other studies.[179, 198, 199]

6.3.2 Importance and limitations of laboratory evidence

From the laboratory results, reaping any beneficial direct antibacterial effects exerted by statins would be confined to topical administration because the MICs for SMV, PTV-lactone, ATV, and FLV against MSSA were over a thousand-fold higher than the respective peak statin plasma concentrations achieved at oral doses consumed for cholesterol-lowering purposes (Section 3.4.1). The topical route of administration offers several advantages over systemic administration such as allowing the administration of high local drug concentrations at the site of infection and reducing systemic toxicity, side effects, and drug interactions with systemic medication.[27] As such, this suggests the topical use of SMV, PTV-lactone, ATV, and FLV may be viable as novel therapeutic agents against SSTIs. However, there are several additional factors to consider.

Firstly, despite minimal systemic absorption, topical antimicrobials are not absolved from the risks of systemic adverse effects because drug transportation occurs through the skin, hair follicle and appendageal glands, and eventually to systemic drug distribution.[27] Drugs with low molecular weight (< 600 Da or < 600 g/mol) due to a high diffusion coefficient permeate the skin better.[238] Hence if statins like FLV, LVS, PRV, PTV, and SMV with molecular weights < 450 g/mol were applied topically, there may be some systemic absorption. Moreover, solvents such as DMSO and alcohols used to dissolve water-insoluble statins are skin penetration enhancers,[239] which further increase the risk of systemic absorption. Given the massive drug

concentrations required to achieve MICs and that drugs administered topically avoid rapid clearance in the gastrointestinal tract or first-pass metabolism (compared to oral administration),[27] the amount of systemic absorption might be sufficient to induce the undesired clinical effects such as diabetes.

Secondly, statins and the three selected metabolites did not exert any antibacterial activity against *E. coli*, *S. marcescens,* and *P. aeruginosa* (Figures 3-3, 3-4, and 3-5 respectively), pathogens which are known to cause complicated SSTIs. It was shown from another study that all seven parent statins do not exert direct antibacterial effects against MRSA as well.[43] Given the lack of activity against pathogens which are implicated in severe SSTIs, the use of statins, including the metabolite PTV-lactone, as novel antibacterial agents appears limited.

Thirdly, obtaining an MIC result at the highest tested concentration of 256 μg/mL does not allow confirmation that the next higher concentration (512 μg/mL) will also demonstrate absence of bacterial growth. This prevents testing for the minimum bactericidal concentration and also precludes detection of a possible paradoxical growth effect for ATV and FLV, which may result in therapeutic failure at higher doses. The anomaly was observed in SMV against MSSA for this study and another study (albeit not discussed),[64] because bacterial growth was noted at drug concentrations higher than the MIC but not at MIC itself. However, the maximum concentration of 256 μg/mL was chosen for this study because this was the highest test concentration recommended in the CLSI guidelines.[67] Moreover, water insoluble statins, such as SMV in our study, would be insoluble in 5% methanol at higher drug concentrations.

Lastly, the use of statins (even as topical agents) could potentially contribute to AMR, since non-antibiotic chemicals with antibacterial properties may induce resistance to multiple antibiotic classes via co-selection (Section 2.4.2), along with statins' ability to cause dysbiosis of the human gut microbiota (Section 2.4.8), statin exposure in bacteraemic patients (Section 2.4.9), and statins' persistence in the environment (Section 2.4.10). The aforementioned considerations collectively diminish any clear advantages of using statins as novel topical therapeutic agents for SSTIs.

By testing the antibacterial effects of all seven statins registered for clinical use and three selected metabolites, a structure-activity relationship analysis could be performed on to postulate a mechanism of action for statins' antibacterial action as a pharmacological class (Section 3.4.2). The suggested mechanism of statins binding with the alanine residues of teichoic acids present on Gram-positive bacterial cell surfaces to reduce biofilm formation, diminish bacterial adhesion to environmental surfaces, or impede *S. aureus* cell division (Section 3.4.3), contributes a fresh perspective to the available literature on statins' plausible mechanisms of antimicrobial activity (Section 2.4.3). Even if statins prove non-viable as novel adjuvants/treatments for SSTIs, the active chemical moieties combining a hydrophobic ring system, lactone ring, and a gem-dimethyl moiety or a cyclopropyl ring may serve as a scaffold for future antibiotic studies (Section 3.4.2).

6.4 Suggestions for future research

Moving forward, evaluating the clinical effects of PTV in a country whereby it is registered for clinical use will help ascertain if PTV may be the only viable statin to serve as a novel adjuvant/treatment for SSTIs. Whilst the search for other novel adjuvants/treatments for SSTIs to serve as AMR breakers and save on significant healthcare resources should continue, the unravelling of statins conceivably contributing to AMR (Section 2.5), despite possessing properties of AMR breakers (Section 1.3.4), remains disturbing.

The use of statins will likely increase with recent guidelines across the world which recommend increased statin use (for primary prevention of cardiovascular diseases) being associated with better outcomes than guidelines advocating reduced statin use.[240] Escalating use of statins would increase the probability of susceptible bacteria being exposed to varying concentrations of statins in humans and the environment, favouring selective pressures or co-selection for AMR (Sections 2.4.2, 2.4.8, 2.4.9, and 2.4.10).

As such, prioritising *in vitro* work to elucidate the statins' antibacterial mechanism of action will provide valuable information because if the antibacterial mechanism

involves directly threatening bacterial survival instead of attenuating virulence factors, AMR is likely to develop more rapidly.[86]

Further to confirming statins' antibacterial mechanism of action, additional research on statins' role in AMR need not preclude work on identifying a novel agent for SSTIs. The human gut microbiome and PXRs are common research areas involving statins and AMR, but also address diabetes, obesity, and infections, which could help in the continued search for novel SSTI treatments.

6.4.1 Research in the human gut microbiome

The human gut microbiota serves as a reservoir of resistant microorganisms.[118] Dysbiosis reduces bacterial diversity in the gut, changing dynamics such as gene expression, protein activity, and overall mechanism, which could result in AMR via the selection for resistant bacteria or new mutations and gene transfers.[118] By disrupting levels of various human gut microbial species such as *Coprococcus comes* and *Ruminococcus torques*,[120] statins potentially promote AMR.[241] Further work could be done to elucidate the specific effects individual statins exert on the gut microbiota, then promote the use of statins with neutral effects on the gut microbiota, or supplement deficient microbiota induced by statins.

Dysbiosis of gut microbiota may also result in increased permeability of the intestinal wall, allowing inflammatory factors such as lipopolysaccharides to pass through and travel through the blood to the liver, adipose tissues, and muscles to develop chronic low-grade inflammation, resulting in metabolic complications such as obesity and diabetes (metabolic endotoxaemia).[242] Homeostasis of the innate and adaptive immune signalling functions is maintained in part via dynamic interactions between the gut microbiota and the intestinal epithelium.[243] Additionally, stimulation of the vagus nerve via the gut microbe-brain-immune axis releases oxytocin which activates Treg cells, conferring wound healing capabilities.[244]

Hence, an effective novel adjuvant to the usual treatment for SSTIs might include the modulation of gut microbiota to avoid obesity and diabetes complications, along with optimising the immune system and wound healing properties.

6.4.2 Research in PXRs

The metabolism and excretion of many clinically used drugs are controlled by PXRs via the regulation of hepatic cytochrome P450 enzymes, organic anion-transporting polypeptides (uptake transporters) in the liver, and P-glycoprotein efflux pumps.[245] Activation of PXR has been associated with tuberculosis drug resistance via drug interactions which reduce the efficacy of anti-tuberculosis drugs, along with increased adverse effects which reduce patient compliance to medication.[245]

Statins such as ATV, LVS, FLV, and LVS have demonstrated significant dose-dependent activation of PXR,[105] which could conceivably contribute towards tuberculosis drug resistance, an emerging global health crisis.[246] Moreover, statin therapy in mice resulted in dysbiosis of gut microbiota via a PXR-dependent mechanism.[121] Hence AMR due to dysbiosis of the gut microbiome via statin-induced activation of PXR is plausible as well. It has been suggested that PXR antagonists might augment the effectiveness of anti-tuberculosis therapy.[247] Thus, future studies could focus on identifying a viable PXR antagonist to supplement anti-tuberculosis drugs and perhaps, statin therapy as well.

The role of PXRs in regulating glucose and lipid metabolism has also been reported.[248] Activation of PXR may increase lipogenesis, causing hepatosteatosis and eventually, diabetes.[248] Conversely, PXR deficiency has been shown to increase energy consumption via amplified utilisation of oxygen and mitochondrial β-oxidation, leading to anti-obesity effects.[248] Incidentally, metformin (an anti-diabetic drug) demonstrated PXR antagonistic effects which are independent of its key anti-diabetic mechanism (activation of adenosine monophosphate-activated protein kinase).[249] Moreover, topical application of metformin was associated with wound healing in rats.[250] As such, future work could examine the viability of metformin and other PXR antagonists as novel adjuvants/treatments for SSTIs, in conjunction with the theoretical potential to stem AMR.

CHAPTER SEVEN

7. Conclusions and Recommendations

Upon reconciling laboratory evidence with clinical evidence between statins and bacterial SSTIs in a translational research framework, it is unlikely that ATV, FLV, PRV, RSV, and SMV may serve as novel adjuvants/treatments for SSTI treatment. The clinical effects of LVS and PTV could not be evaluated as they are not registered for clinical use in Australia. However, based on laboratory work in this study and clinical effects reported in other literature, oral PTV is possibly the only clinically used statin with potential to be a novel topical agent for SSTIs due to its favourable systemic effects on diabetes via increasing adiponectin levels. Although SMV, PTV-lactone, ATV, and FLV demonstrated *in vitro* antibacterial effects against MSSA, the combined possibility of systemic absorption (due to massive drug concentrations required to achieve MICs), lack of antibacterial activity against pathogens causing severe SSTIs, and risk of statin contribution to AMR collectively do not support the use of statins as topical novel therapeutic agents for SSTIs.

Statins are theoretically ideal as novel agents to reduce AMR and treat SSTIs due to a multipronged approach of possessing direct antibacterial activity against MRSA and pathogens such as *E. coli*, *Enterococcus,* and *Streptococcus* species which cause SSTIs, synergise with topical antimicrobials against MRSA, stimulate the human immune system, modulate sepsis via anti-inflammatory effects, promote wound healing, and suppress bacterial toxins.

However, the outpatient clinical evidence gathered showed that statins may increase the risk of SSTIs through a direct mechanism (reduction of innate immunity) or through an indirect mechanism (increasing the risk of diabetes, in turn a risk factor for SSTIs). Inpatient clinical evidence demonstrated neither significant benefits of statin use on the risk of SSTIs nor better clinical outcomes compared to non-statin users. Instead, patients with SSTIs using ATV were associated with a significantly increased risk of diabetes, which being a risk factor for *S. aureus*-related SSTIs, would likely progress to recurrent SSTIs.

Viewing the outpatient and inpatient data collectively, the use of ATV, FLV, PRV, RSV, and SMV was paradoxically associated with an increased risk of SSTIs likely because of its small but significant association with diabetes. The sensitive period of greatest diabetes risk was found to be as short as 91 days after statin commencement, predisposing patients to *S. aureus*-related SSTIs, funnelling into a vicious cycle of recurrent SSTIs. Hence, it may be advisable for physicians to regularly monitor the blood glucose levels of patients on statins and be mindful of SSTI risks.

Given the lack of support from laboratory evidence for topical statin use and risks of diabetes from clinical evidence, further research on the clinical effects of PTV in a country where it is registered for clinical use would help confirm whether oral PTV will emerge as the only statin viable for repurposing as a novel systemic therapeutic agents against SSTIs. Nevertheless, the identification of chemical moieties likely responsible for statins' antibacterial activity (i.e. combination of a hydrophobic statin ring system, a lactone ring, and a gem-dimethyl moiety or a cyclopropyl ring), may serve as a scaffold for future antibiotic studies.

Of greater concern, despite statins possessing theoretical properties which impede AMR, the exposure of susceptible bacteria to varying concentrations of statins within the human body and in the environment may instead, favour selective pressures for bacterial resistance or co-selection for resistant genes. With various countries' guidelines affirming statin use for preventing cardiovascular diseases, the already extensive use of statins will likely be expanded, potentially compounding the AMR crisis. Prioritising research to elucidate statins' antibacterial mechanism of action might be of greatest value to determine the imminence of statin-associated AMR, especially if the true mechanism directly threatens bacteria survival.

The evidence presented in this thesis not only refuted the initial hypothesis of repurposing statins in general as novel therapeutic agents for SSTIs to help curb AMR, it also revealed the ominous possibility that statins may be associated with AMR instead. It is hoped that the postulated mechanism of statins' antibacterial action and suggested areas of research in the human gut microbiome and PXRs, amongst other contributions, might support and invoke further research in the search

for other novel SSTI treatments, in tandem with addressing statins' influence on AMR.

REFERENCES

1. Lai EC, Pratt N, Hsieh CY, Lin SJ, Pottegard A, Roughead EE, et al. Sequence symmetry analysis in pharmacovigilance and pharmacoepidemiologic studies. Eur J Epidemiol. 2017;32:567-82. doi:10.1007/s10654-017-0281-8.

2. World Health Organization. Antimicrobial resistance: global report on surveillance 2014. Geneva 2014 [cited 2016 Sep 01]. Available from: http://www.who.int/drugresistance/documents/surveillancereport/en/.

3. Rangel-Vega A, Bernstein LR, Mandujano-Tinoco EA, Garcia-Contreras SJ, Garcia-Contreras R. Drug repurposing as an alternative for the treatment of recalcitrant bacterial infections. Front Microbiol. 2015;6:Article 282. doi:10.3389/fmicb.2015.00282.

4. Chong CR, Sullivan DJ, Jr. New uses for old drugs. Nature. 2007;448:645-6. doi:10.1038/448645a.

5. Brown D. Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void? Nat Rev Drug Discov. 2015;14:821-32. doi:10.1038/nrd4675.

6. Oprea TI, Bauman JE, Bologa CG, Buranda T, Chigaev A, Edwards BS, et al. Drug repurposing from an academic perspective. Drug Discov Today Ther Strateg. 2011;8:61-9. doi:10.1016/j.ddstr.2011.10.002.

7. Blaha MJ, Martin SS. How do statins work? Changing paradigms with implications for statin allocation. J Am Coll Cardiol. 2013;62:2392-4. doi:10.1016/j.jacc.2013.08.1626.

8. Ki V, Rotstein C. Bacterial skin and soft tissue infections in adults: A review of their epidemiology, pathogenesis, diagnosis, treatment and site of care. Can J Infect Dis Med Microbiol. 2008;19:173-84. doi:10.1155/2008/846453.

9. Pretorius J. Skin and soft-tissue infections: The early clinical presentation of soft-tissue infections may be deceptive. Continuing Medical Education. 2010;28:265-9.

10. Miller LS, Cho JS. Immunity against *Staphylococcus aureus* cutaneous infections. Nat Rev Immunol. 2011;11:505-18. doi:10.1038/nri3010.

11. Creech CB, Al-Zubeidi DN, Fritz SA. Prevention of recurrent staphylococcal skin infections. Infect Dis Clin North Am. 2015;29:429-64. doi:10.1016/j.idc.2015.05.007.

12. Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol. 2011;9:244-53. doi:10.1038/nrmicro2537.

13. Tognetti L, Martinelli C, Berti S, Hercogova J, Lotti T, Leoncini F, et al. Bacterial skin and soft tissue infections: Review of the epidemiology, microbiology, aetiopathogenesis and treatment: A collaboration between dermatologists and infectivologists. J Eur Acad Dermatol Venereol. 2012;26:931-41. doi:10.1111/j.1468-3083.2011.04416.x.

14. Ramakrishnan K, Salinas RC, Agudelo Higuita NI. Skin and soft tissue infections. Am Fam Physician. 2015;92:474-83

15. Palit A, Inamadar AC. Current concepts in the management of bacterial skin infections in children. Indian J Dermatol Venereol Leprol. 2010;76:476-88. doi:10.4103/0378-6323.69053.

16. Petkovsek Z, Elersic K, Gubina M, Zgur-Bertok D, Starcic Erjavec M. Virulence potential of *Escherichia coli* isolates from skin and soft tissue infections. J Clin Microbiol. 2009;47:1811-7. doi:10.1128/JCM.01421-08.

17. Biscoe A, Hakeem L. Severe soft tissue infection in a patient with type 2 diabetes mellitus caused by *Serratia marcescens* as single pathogen. Br J Diabetes. 2016;16:202-5. doi:10.15277/bjd.2016.111.

18. Ali A, Botha J, Tiruvoipati R. Fatal skin and soft tissue infection of multidrug resistant *Acinetobacter baumannii*: A case report. Int J Surg Case Rep. 2014;5:532-6. doi:10.1016/j.ijscr.2014.04.019.

19. McClain SL, Bohan JG, Stevens DL. Advances in the medical management of skin and soft tissue infections. BMJ. 2016;355:i6004. doi:10.1136/bmj.i6004.

20. Dryden MS. Alternative clinical indications for novel antibiotics licensed for skin and soft tissue infection? Curr Opin Infect Dis. 2015;28:117-24. doi:10.1097/QCO.0000000000000142.

21. Stevens DL, Bisno AL, Chambers HF, Dellinger EP, Goldstein EJ, Gorbach SL, et al. Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the Infectious Diseases Society of America. Clin Infect Dis. 2014;59:e10-52. doi:10.1093/cid/ciu444.

22. Montravers P, Snauwaert A, Welsch C. Current guidelines and recommendations for the management of skin and soft tissue infections. Curr Opin Infect Dis. 2016;29:131-8. doi:10.1097/QCO.0000000000000242.

23. Raff AB, Kroshinsky D. Cellulitis: a review. JAMA. 2016;316:325-37. doi:10.1001/jama.2016.8825.

24. Kaye KS, Patel DA, Stephens JM, Khachatryan A, Patel A, Johnson K. Rising United States hospital admissions for acute bacterial skin and skin structure infections: recent trends and economic impact. PLoS One. 2015;10:e0143276. doi:10.1371/journal.pone.0143276.

25. Hurley HJ, Knepper BC, Price CS, Mehler PS, Burman WJ, Jenkins TC. Avoidable antibiotic exposure for uncomplicated skin and soft tissue infections in the ambulatory care setting. Am J Med. 2013;126:1099-106. doi:10.1016/j.amjmed.2013.08.016.

26. Lee CR, Cho IH, Jeong BC, Lee SH. Strategies to minimize antibiotic resistance. Int J Environ Res Public Health. 2013;10:4274-305. doi:10.3390/ijerph10094274.

27. Lam PL, Lee KKH, Wong RSM, Cheng GYM, Bian ZX, Chui CH, et al. Recent advances on topical antimicrobials for skin and soft tissue infections and their safety concerns. Crit Rev Microbiol. 2017;44:40-78. doi:10.1080/1040841X.2017.1313811.

28. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG, Jr. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev. 2015;28:603-61. doi:10.1128/CMR.00134-14.

29. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48:1-12. doi:10.1086/595011.

30. Basak S, Singh P, Rajurkar M. Multidrug resistant and extensively drug resistant bacteria: a study. J Pathog. 2016;2016:4065603. doi:10.1155/2016/4065603.

31. Wright GD. Something old, something new: revisiting natural products in antibiotic drug discovery. Can J Microbiol. 2014;60:147-54. doi:10.1139/cjm-2014-0063.

32. Shlaes DM, Sahm D, Opiela C, Spellberg B. The FDA reboot of antibiotic development. Antimicrob Agents Chemother. 2013;57:4605-7. doi:10.1128/AAC.01277-13.

33. Piddock LJ. The crisis of no new antibiotics - what is the way forward? Lancet Infect Dis. 2012;12:249-53. doi:10.1016/S1473-3099(11)70316-4.

34. Coates AR, Halls G, Hu Y. Novel classes of antibiotics or more of the same? Br J Pharmacol. 2011;163:184-94. doi:10.1111/j.1476-5381.2011.01250.x.

35. Livermore DM, on behalf of the British Society for Antimicrobial Chemotherapy Working Party on the urgent need: Regenerating antibacterial drug discovery and development. Discovery research: the scientific challenge of finding new antibiotics. J Antimicrob Chemother. 2011;66:1941-4. doi:10.1093/jac/dkr262.

36. Collins R, Reith C, Emberson J, Armitage J, Baigent C, Blackwell L, et al. Interpretation of the evidence for the efficacy and safety of statin therapy. Lancet. 2016;388:2532-61. doi:10.1016/S0140-6736(16)31357-5.

37. Liao KJ. Clinical implications for statin pleiotropy. Curr Opin Lipidol. 2005;16:624-9. doi:10.1097/01.mol.0000191913.16321.60.

38. Gazzerro P, Proto MC, Gangemi G, Malfitano AM, Ciaglia E, Pisanti S, et al. Pharmacological actions of statins: a critical appraisal in the management of cancer. Pharmacol Rev. 2012;64:102-46. doi:10.1124/pr.111.004994.

39. Kozarov E, Padro T, Badimon L. View of statins as antimicrobials in cardiovascular risk modification. Cardiovasc Res. 2014;102:362-74. doi:10.1093/cvr/cvu058.

40. Chow OA, von Kockritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL, et al. Statins enhance formation of phagocyte extracellular traps. Cell Host Microbe. 2010;8:445-54. doi:10.1016/j.chom.2010.10.005.

41. Fitzmaurice GJ, McWilliams B, Nolke L, Redmond JM, McGuinness JG, O'Donnell ME. Do statins have a role in the promotion of postoperative wound healing in cardiac surgical patients? Ann Thorac Surg. 2014;98:756-64. doi:10.1016/j.athoracsur.2014.02.089.

42. Jerwood S, Cohen J. Unexpected antimicrobial effect of statins. J Antimicrob Chemother. 2008;61:362-4. doi:10.1093/jac/dkm496.

43. Thangamani S, Mohammad H, Abushahba MF, Hamed MI, Sobreira TJ, Hedrick VE, et al. Exploring simvastatin, an antihyperlipidemic drug, as a potential topical antibacterial agent. Sci Rep. 2015;5:16407. doi:10.1038/srep16407.

44. Masadeh M, Mhaidat N, Alzoubi K, Al-Azzam S, Alnasser Z. Antibacterial activity of statins: a comparative study of atorvastatin, simvastatin, and rosuvastatin. Ann Clin Microbiol Antimicrob. 2012;11:13. doi:10.1186/1476-0711-11-13.

45. Terblanche M, Almog Y, Rosenson RS, Smith TS, Hackam DG. Statins and sepsis: multiple modifications at multiple levels. Lancet Infect Dis. 2007;7:358-68. doi:10.1016/S1473-3099(07)70111-1.

46. Hennessy E, Adams C, Reen FJ, O'Gara F. Is there potential for repurposing statins as novel antimicrobials? Antimicrob Agents Chemother. 2016;60:5111-21. doi:10.1128/AAC.00192-16.

47. Ko HHT, Lareu RR, Dix BR, Hughes JD. Statins: antimicrobial resistance breakers or makers? PeerJ. 2017;5:e3952. doi:10.7717/peerj.3952.

48. Creative Commons. Attribution 4.0 International Public License (CC-BY 4.0). [Internet]. [cited 9 May 2018]. Available from: https://creativecommons.org/licenses/by/4.0/.

49. Dafale NA, Semwal UP, Rajput RK, Singh GN. Selection of appropriate analytical tools to determine the potency and bioactivity of antibiotics and antibiotic resistance. J Pharm Anal. 2016;6:207-13. doi:http://dx.doi.org/10.1016/j.jpha.2016.05.006.

50. Andersson DI, Hughes D. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol Rev. 2011;35:901-11. doi:10.1111/j.1574-6976.2011.00289.x.

51. Levison ME, Levison JH. Pharmacokinetics and pharmacodynamics of antibacterial agents. Infect Dis Clin North Am. 2009;23:791-815. doi:10.1016/j.idc.2009.06.008.

52. Tobert JA. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. Nat Rev Drug Discov. 2003;2:517-26. doi:10.1038/nrd1112.

53. Alshammari A. *In vitro* effect of statins on *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius*: Temple University, Philadelphia, United States of America; 2016.

54. Bjorkhem-Bergman L, Lindh JD, Bergman P. What is a relevant statin concentration in cell experiments claiming pleiotropic effects? Br J Clin Pharmacol. 2011;72:164-5. doi:10.1111/j.1365-2125.2011.03907.x.

55. Coban AY, Tekeli HO, Guney AK, Durupinar B. [Investigation of the *in vitro* antibacterial effects of statins] [Article in Turkish]. Mikrobiyol Bul. 2010;44:161-3.

56. Quivey R. Reducing dental caries. Google Patents: University Of Rochester 2014 [cited 2016 Dec 08]. Available from: https://www.google.com/patents/US20140186271.

57. Ting M, Whitaker EJ, Albandar JM. Systematic review of the *in vitro* effects of statins on oral and perioral microorganisms. Eur J Oral Sci. 2016;124:4-10. doi:10.1111/eos.12239.

58. Bergman P, Linde C, Putsep K, Pohanka A, Normark S, Henriques-Normark B, et al. Studies on the antibacterial effects of statins - *in vitro* and *in vivo*. PLoS One. 2011;6:e24394. doi:10.1371/journal.pone.0024394.

59. Emani S, Gunjiganur GV, Mehta DS. Determination of the antibacterial activity of simvastatin against periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*: An *in vitro* study. Contemp Clin Dent. 2014;5:377-82. doi:10.4103/0976-237X.137959.

60. Graziano TS, Cuzzullin MC, Franco GC, Schwartz-Filho HO, de Andrade ED, Groppo FC, et al. Statins and antimicrobial effects: Simvastatin as a potential drug against *Staphylococcus aureus* biofilm. PLoS One. 2015;10:e0128098. doi:10.1371/journal.pone.0128098.

61. Matzneller P, Manafi M, Zeitlinger M. Antimicrobial effect of statins: organic solvents might falsify microbiological testing results. Int J Clin Pharmacol Ther. 2011;49:666-71. doi:10.5414/CP201581.

62. Radwan S, Ezzat O. Antimicrobial effect and immunomodulation of atorvastatin. J Am Sci. 2012;8:1012-16.

63. Sarabhai S, Dhaliwal LK, Capalash N, Sharma P. Effect of atorvastatin and rosuvastatin on quorum sensing, biofilm formation and bacterial motilities of *Pseudomonas aeruginosa*. Int J Pharma Bio Sci. 2015;6:(B) 1-8.

64. Wang CC, Yang PW, Yang SF, Hsieh KP, Tseng SP, Lin YC. Topical simvastatin promotes healing of *Staphylococcus aureus* - contaminated cutaneous wounds. Int Wound J. 2016;13:1150-7. doi:10.1111/iwj.12431.

65. Welsh AM, Kruger P, Faoagali J. Antimicrobial action of atorvastatin and rosuvastatin. Pathology. 2009;41:689-91. doi:10.3109/00313020903305860.

66. Turnidge J, Paterson DL. Setting and revising antibacterial susceptibility breakpoints. Clin Microbiol Rev. 2007;20:l391-408. doi:10.1128/CMR.00047-06.

67. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard-ninth edition. CLSI document M07-A9. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute; 2012.

68. Liu M, Lu J, Muller P, Turnbull L, Burke CM, Schlothauer RC, et al. Antibiotic-specific differences in the response of *Staphylococcus aureus* to treatment with antimicrobials combined with manuka honey. Front Microbiol. 2015;5:Article 779. doi:10.3389/fmicb.2014.00779.

69. Shanholtzer CJ, Peterson LR, Mohn ML, Moody JA, Gerding DN. MBCs for *Staphylococcus aureus* as determined by macrodilution and microdilution techniques. Antimicrob Agents Chemother. 1984;26:214-9. doi:10.1128/AAC.26.2.214

70. Hughes D, Andersson DI. Evolutionary trajectories to antibiotic resistance. Annu Rev Microbiol. 2017;71:579-96. doi:10.1146/annurev-micro-090816-093813.

71. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015;13:42-51. doi:10.1038/nrmicro3380.

72. Singer AC, Shaw H, Rhodes V, Hart A. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. Front Microbiol. 2016;7:Article 1728. doi:10.3389/fmicb.2016.01728.

73. Wales AD, Davies RH. Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. Antibiotics (Basel). 2015;4:567-604. doi:10.3390/antibiotics4040567.

74. Li D, Zeng S, He M, Gu AZ. Water disinfection byproducts induce antibiotic resistance - role of environmental pollutants in resistance phenomena. Environ Sci Technol. 2016;50:3193-201. doi:10.1021/acs.est.5b05113.

75. Laudy A, Kulińska E, Tyski S. The impact of efflux pump inhibitors on the activity of selected non-antibiotic medicinal products against Gram-negative bacteria. Molecules. 2017;22:114. doi:10.3390/molecules22010114.

76. Haeri MR, White K, Qharebeglou M, Ansar MM. Cholesterol suppresses antimicrobial effect of statins. Iran J Basic Med Sci. 2015;18:1253-6

77. Endo A. A historical perspective on the discovery of statins. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86:484-93

78. Harrold M. Antihyperlipoproteinemics and inhibitors of cholesterol biosynthesis. In: Lemke TL, Williams DA, Roche VF, Zito SW, editors. Foye's Principles of Medicinal Chemistry. 7 ed. Philadelphia, United States of America: Lippincott Williams & Wilkins; 2013.

79. Liao JK, Laufs U. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol. 2005;45:89-118. doi:10.1146/annurev.pharmtox.45.120403.095748.

80. Armitage J. The safety of statins in clinical practice. Lancet. 2007;370:1781-90. doi:10.1016/S0140-6736(07)60716-8.

81. Heuston S, Begley M, Gahan CG, Hill C. Isoprenoid biosynthesis in bacterial pathogens. Microbiology. 2012;158:1389-401. doi:10.1099/mic.0.051599-0.

82. Friesen JA, Rodwell VW. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol. 2004;5:248. doi:10.1186/gb-2004-5-11-248.

83. Brooks BD, Brooks AE. Therapeutic strategies to combat antibiotic resistance. Adv Drug Deliv Rev. 2014;78:14-27. doi:10.1016/j.addr.2014.10.027.

84. Farmer AR, Murray CK, Mende K, Akers KS, Zera WC, Beckius ML, et al. Effect of HMG-CoA reductase inhibitors on antimicrobial susceptibilities for Gram-negative rods. J Basic Microbiol. 2013;53:336-9. doi:10.1002/jobm.201100614.

85. Wu HJ, Wang AH, Jennings MP. Discovery of virulence factors of pathogenic bacteria. Curr Opin Chem Biol. 2008;12:93-101. doi:10.1016/j.cbpa.2008.01.023.

86. Park B, Liu GY. Targeting the host-pathogen interface for treatment of *Staphylococcus aureus* infection. Semin Immunopathol. 2012;34:299-315. doi:10.1007/s00281-011-0297-1.

87. Parihar SP, Hartley MA, Hurdayal R, Guler R, Brombacher F. Topical simvastatin as host-directed therapy against aeverity of cutaneous leishmaniasis in mice. Sci Rep. 2016;6:33458. doi:10.1038/srep33458.

88. Frostegård J, Zhang Y, Sun J, Yan K, Liu A. Oxidized low-density lipoprotein (OxLDL) – treated dendritic cells promote activation of T cells in human atherosclerotic plaque and blood, which is repressed by statins: microRNA let-7c is integral to the effect. J Am Heart Assoc. 2016;5:e003976. doi:10.1161/jaha.116.003976.

89. Walton GM, Stockley JA, Griffiths D, Sadhra CS, Purvis T, Sapey E. Repurposing treatments to enhance innate immunity. Can statins improve neutrophil functions and clinical outcomes in COPD? J Clin Med. 2016;5:89. doi:10.3390/jcm5100089.

90. Yang Z, Huang YC, Koziel H, de Crom R, Ruetten H, Wohlfart P, et al. Female resistance to pneumonia identifies lung macrophage nitric oxide synthase-3 as a therapeutic target. Elife. 2014;3. doi:10.7554/eLife.03711.

91. Frostegard J, Zhang Y, Sun J, Yan K, Liu A. Oxidized Low-Density Lipoprotein (OxLDL)-Treated Dendritic Cells Promote Activation of T Cells in Human Atherosclerotic Plaque and Blood, Which Is Repressed by Statins: microRNA let-7c Is Integral to the Effect. J Am Heart Assoc. 2016;5. doi:10.1161/JAHA.116.003976.

92. Sorensen OE, Borregaard N. Neutrophil extracellular traps - the dark side of neutrophils. J Clin Invest. 2016;126:1612-20. doi:10.1172/JCI84538.

93. Millar PJ, Floras JS. Statins and the autonomic nervous system. Clin Sci (Lond). 2014;126:401-15. doi:10.1042/CS20130332.

94. Wittebole X, Castanares-Zapatero D, Laterre PF. Toll-like receptor 4 modulation as a strategy to treat sepsis. Mediators Inflamm. 2010;2010:568396. doi:10.1155/2010/568396.

95. Vera S, Martinez R, Gormaz JG, Gajardo A, Galleguillos F, Rodrigo R. Novel relationships between oxidative stress and angiogenesis-related factors in sepsis: New biomarkers and therapies. Ann Med. 2015;47:289-300. doi:10.3109/07853890.2015.1029967.

96. Janda S, Young A, Fitzgerald JM, Etminan M, Swiston J. The effect of statins on mortality from severe infections and sepsis: a systematic review and meta-analysis. J Crit Care. 2010;25:656.e7–e22. doi:10.1016/j.jcrc.2010.02.013.

97. Bjorkhem-Bergman L, Bergman P, Andersson J, Lindh JD. Statin treatment and mortality in bacterial infections - a systematic review and meta-analysis. PLoS One. 2010;5:e10702. doi:10.1371/journal.pone.0010702.

98. Deshpande A, Pasupuleti V, Rothberg MB. Statin therapy and mortality from sepsis: a meta-analysis of randomized trials. Am J Med. 2015;128:410-7. doi:10.1016/j.amjmed.2014.10.057.

99. Quinn M, Moody C, Tunnicliffe B, Khan Z, Manji M, Gudibande S, et al. Systematic review of statins in sepsis: There is no evidence of dose response. Indian J Crit Care Med. 2016;20:534-41. doi:10.4103/0972-5229.190366.

100. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. Nat Rev Immunol. 2008;8:776-87. doi:10.1038/nri2402.

101. Murphy TJ, Paterson HM, Mannick JA, Lederer JA. Injury, sepsis, and the regulation of Toll-like receptor responses. J Leukoc Biol. 2004;75:400-7. doi:10.1189/jlb.0503233.

102. Weinstein LI, Revuelta A, Pando RH. Catecholamines and acetylcholine are key regulators of the interaction between microbes and the immune system. Ann N Y Acad Sci. 2015;1351:39-51. doi:10.1111/nyas.12792.

103. Binkowska AM, Michalak G, Slotwinski R. Current views on the mechanisms of immune responses to trauma and infection. Cent Eur J Immunol. 2015;40:206-16. doi:10.5114/ceji.2015.52835.

104. Ou SY, Chu H, Chao PW, Ou SM, Lee YJ, Kuo SC, et al. Effect of the use of low and high potency statins and sepsis outcomes. Intensive Care Med. 2014;40:1509-17. doi:10.1007/s00134-014-3418-1.

105. Howe K, Sanat F, Thumser AE, Coleman T, Plant N. The statin class of HMG-CoA reductase inhibitors demonstrate differential activation of the nuclear

receptors PXR, CAR and FXR, as well as their downstream target genes. Xenobiotica. 2011;41:519-29. doi:10.3109/00498254.2011.569773.

106. Marshall TG. Are statins analogues of vitamin D? Lancet. 2006;368:1234; author reply 5. doi:10.1016/S0140-6736(06)69509-3.

107. Paumelle R, Staels B. Peroxisome proliferator-activated receptors mediate pleiotropic actions of statins. Circ Res. 2007;100:1394-5. doi:10.1161/01.RES.0000269334.42814.d2.

108. Schaap FG, Trauner M, Jansen PL. Bile acid receptors as targets for drug development. Nat Rev Gastroenterol Hepatol. 2014;11:55-67. doi:10.1038/nrgastro.2013.151.

109. Kadmiel M, Cidlowski JA. Glucocorticoid receptor signaling in health and disease. Trends Pharmacol Sci. 2013;34:518-30. doi:10.1016/j.tips.2013.07.003.

110. Gombart AF. The vitamin D-antimicrobial peptide pathway and its role in protection against infection. Future Microbiol. 2009;4:1151-65. doi:10.2217/fmb.09.87.

111. Mangin M, Sinha R, Fincher K. Inflammation and vitamin D: the infection connection. Inflamm Res. 2014;63:803-19. doi:10.1007/s00011-014-0755-z.

112. Reen FJ, Flynn S, Woods DF, Dunphy N, Chroinin MN, Mullane D, et al. Bile signalling promotes chronic respiratory infections and antibiotic tolerance. Sci Rep. 2016;6:29768. doi:10.1038/srep29768.

113. Bu DX, Griffin G, Lichtman AH. Mechanisms for the anti-inflammatory effects of statins. Curr Opin Lipidol. 2011;22:165-70. doi:10.1097/MOL.0b013e3283453e41.

114. Elewa HF, El-Remessy AB, Somanath PR, Fagan SC. Diverse effects of statins on angiogenesis: new therapeutic avenues. Pharmacotherapy. 2010;30:169-76. doi:10.1592/phco.30.2.169.

115. Farsaei S, Khalili H, Farboud ES. Potential role of statins on wound healing: review of the literature. Int Wound J. 2012;9:238-47. doi:10.1111/j.1742-481X.2011.00888.x.

116. Vukelic S, Stojadinovic O, Pastar I, Vouthounis C, Krzyzanowska A, Das S, et al. Farnesyl pyrophosphate inhibits epithelialization and wound healing through the glucocorticoid receptor. J Biol Chem. 2010;285:1980-8. doi:10.1074/jbc.M109.016741.

117. Calanni F, Renzulli C, Barbanti M, Viscomi GC. Rifaximin: beyond the traditional antibiotic activity. J Antibiot (Tokyo). 2014;67:667-70. doi:10.1038/ja.2014.106.

118. Francino MP. Antibiotics and the human gut microbiome: Dysbioses and accumulation of resistances. Front Microbiol. 2016;6:Article 1543. doi:10.3389/fmicb.2015.01543.

119. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science. 2016;352:565-9. doi:10.1126/science.aad3369.

120. Le Bastard Q, Al-Ghalith GA, Gregoire M, Chapelet G, Javaudin F, Dailly E, et al. Systematic review: human gut dysbiosis induced by non-antibiotic prescription medications. Aliment Pharmacol Ther. 2018;47:332-45. doi:10.1111/apt.14451.

121. Caparros-Martin JA, Lareu RR, Ramsay JP, Peplies J, Jerry Reen F, Headlam HA, et al. Statin therapy causes gut dysbiosis in mice through a PXR-dependent mechanism. Microbiome. 2017;5:95. doi:10.1186/s40168-017-0312-4.

122. Khutoryanskiy VV. Supramolecular materials: Longer and safer gastric residence. Nat Mater. 2015;14:963-4. doi:10.1038/nmat4432.

123. McFarland AJ, Anoopkumar-Dukie S, Arora DS, Grant GD, McDermott CM, Perkins AV, et al. Molecular mechanisms underlying the effects of statins in the central nervous system. Int J Mol Sci. 2014;15:20607-37. doi:10.3390/ijms151120607.

124. Reinoso RF, Sanchez Navarro A, Garcia MJ, Prous JR. Preclinical pharmacokinetics of statins. Methods Find Exp Clin Pharmacol. 2002;24:593-613.

125. Kantola T, Backman JT, Niemi M, Kivisto KT, Neuvonen PJ. Effect of fluconazole on plasma fluvastatin and pravastatin concentrations. Eur J Clin Pharmacol. 2000;56:225-9. doi:10.1007/s002280000127.

126. Lee HB, Peart TE, Svoboda ML, Backus S. Occurrence and fate of rosuvastatin, rosuvastatin lactone, and atorvastatin in Canadian sewage and surface water samples. Chemosphere. 2009;77:1285-91. doi:10.1016/j.chemosphere.2009.09.068.

127. Ottmar KJ, Colosi LM, Smith JA. Fate and transport of atorvastatin and simvastatin drugs during conventional wastewater treatment. Chemosphere. 2012;88:1184-9. doi:10.1016/j.chemosphere.2012.03.066.

128. Ramana KV, Pinnelli VB, Prakash B, CR WDS, Kandi S, Sharada CV, et al. Complicated skin and skin structure infections (cSSSI's): A comprehensive review. American Journal of Medical and Biological Research. 2013;1:159-64. doi:10.12691/ajmbr-1-4-9.

129. Betteridge DJ, Carmena R. The diabetogenic action of statins - mechanisms and clinical implications. Nat Rev Endocrinol. 2016;12:99-110. doi:10.1038/nrendo.2015.194.

130. Ko HHT, Lareu RR, Dix BR, Hughes JD. *In vitro* antibacterial effects of statins against bacterial pathogens causing skin infections. Eur J Clin Microbiol Infect Dis. 2018;37:1125-35. doi:10.1007/s10096-018-3227-5.

131. Brown ED, Wright GD. Antibacterial drug discovery in the resistance era. Nature. 2016;529:336-43. doi:10.1038/nature17042.

132. Basch H, Gadebusch HH. *In vitro* antimicrobial activity of dimethylsulfoxide. Appl Microbiol. 1968;16:1953-4.

133. Yamashita M, Takeno A. Relationship between bactericidal activity and the hydrophobicity - Hydrophilicity balance of alcohol solutions. Biocontrol Sci. 2001;6:107-11.

134. Kim S, Kim J, Lim W, Jeon S, Kim O, Koh JT, et al. *In vitro* bactericidal effects of 625, 525, and 425 nm wavelength (red, green, and blue) light-emitting diode irradiation. Photomed Laser Surg. 2013;31:554-62. doi:10.1089/pho.2012.3343.

135. Zhou Q, Chen QX, Ruan ZR, Yuan H, Xu HM, Zeng S. CYP2C9*3(1075A > C), ABCB1 and SLCO1B1 genetic polymorphisms and gender are determinants of inter-subject variability in pitavastatin pharmacokinetics. Pharmazie. 2013;68:187-94. doi:10.1691/ph.2013.2742.

136. Causevic-Ramosevac A, Semiz S. Drug interactions with statins. Acta Pharm. 2013;63:277-93. doi:10.2478/acph-2013-0022.

137. Duimel-Peeters I, Houwing R, Teunissen C, Berger M, Snoeckx L, Halfens R. A systematic review of the efficacy of topical skin application of dimethyl sulfoxide on wound healing and as an anti-inflammatory. Wounds. 2003;15:316-70.

138. Redelman CV, Maduakolam C, Anderson GG. Alcohol treatment enhances *Staphylococcus aureus* biofilm development. FEMS Immunol Med Microbiol. 2012;66:411-8. doi:10.1111/1574-695X.12005.

139. Korem M, Gov Y, Shirron N, Shuster A, Rosenberg M. Alcohol increases hemolysis by staphylococci. FEMS Microbiol Lett. 2007;269:153-9. doi:10.1111/j.1574-6968.2006.00625.x.

140. Boland T, Latour RA, Stutzenberger FJ. Molecular basis of bacterial adhesion. In: Yuehuei HA, Friedman RJ, editors. Handbook of bacterial adhesion: Principles, methods, and applications. 2000 ed. Totowa, New Jersey: Humana Press Inc; 2000. p. 29-41.

141. Chen KX, Njoroge FG. NS3 protease covalent inhibitors. In: Tan SL, He Y, editors. Hepatitis C: antiviral drug discovery and development. United Kingdom: Caister Academic Press; 2011. p. 169-92.

142. Brown S, Santa Maria JP, Jr., Walker S. Wall teichoic acids of Gram-positive bacteria. Annu Rev Microbiol. 2013;67:313-36. doi:10.1146/annurev-micro-092412-155620.

143. Shi JH, Wang Q, Pan DQ, Liu TT, Jiang M. Characterization of interactions of simvastatin, pravastatin, fluvastatin, and pitavastatin with bovine serum albumin: multiple spectroscopic and molecular docking. J Biomol Struct Dyn. 2017;35:1529-46. doi:10.1080/07391102.2016.1188416.

144. Hanson BR, Neely MN. Coordinate regulation of Gram-positive cell surface components. Curr Opin Microbiol. 2012;15:204-10. doi:10.1016/j.mib.2011.12.011.

145. Foster TJ, Geoghegan JA, Ganesh VK, Hook M. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol. 2014;12:49-62. doi:10.1038/nrmicro3161.

146. Shi JH, Wang Q, Pan DQ, Liu TT, Jiang M. Characterization of interactions of simvastatin, pravastatin, fluvastatin, and pitavastatin with bovine serum albumin: multiple spectroscopic and molecular docking. J Biomol Struct Dyn. 2016:1-18. doi:10.1080/07391102.2016.1188416.

147. Kruszewska H, Zareba T, Tyski S. Examination of antimicrobial activity of selected non-antibiotic drugs. Acta Pol Pharm. 2004;61 Suppl:18-21.

148. Kocsis E, Kristóf K, Hermann P, Rozgonyi F. A comparative review on the pathogenicity and virulence factors of meticillin-resistant and meticillin-susceptible *Staphylococcus aureus*. Rev Med Microbiol. 2010;21:31-7. doi:10.1097/MRM.0b013e3283393cd4.

149. Shine WE, Silvany R, McCulley JP. Relation of cholesterol-stimulated *Staphylococcus aureus* growth to chronic blepharitis. Invest Ophthalmol Vis Sci. 1993;34:2291-6.

150. Vanstraelen K, Lagrou K, Maertens J, Wauters J, Willems L, Spriet I. The Eagle-like effect of echinocandins: what's in a name? Expert Rev Anti Infect Ther. 2013;11:1179-91. doi:10.1586/14787210.2013.841543.

151. Holm SE, Tornqvist IO, Cars O. Paradoxical effects of antibiotics. Scand J Infect Dis. 1991;22 (Suppl.74):113-7.

152. Ferreira JA, Carr JH, Starling CE, de Resende MA, Donlan RM. Biofilm formation and effect of caspofungin on biofilm structure of Candida species bloodstream isolates. Antimicrob Agents Chemother. 2009;53:4377-84. doi:10.1128/AAC.00316-09.

153. Stover KR, Cleary JD. The Eagle-like effect of the echinocandins: is it relevant for clinical decisions? Curr Fungal Infect Rep. 2015;9:88-93. doi:10.1007/s12281-015-0221-6.

154. Tanouchi Y, Pai A, Buchler NE, You L. Programming stress-induced altruistic death in engineered bacteria. Mol Syst Biol. 2012;8:626. doi:10.1038/msb.2012.57.

155. Smit J, Lopez-Cortes LE, Thomsen RW, Schonheyder HC, Nielsen H, Froslev T, et al. Statin use and risk of community-acquired *Staphylococcus aureus* bacteremia: a population-based case-control study. Mayo Clin Proc. 2017;92:1469-78. doi:10.1016/j.mayocp.2017.07.008.

156. Weintrob AC, Sexton DJ. Susceptibility to infections in persons with diabetes mellitus. 2016. In: UpToDate [Internet]. UpToDate, Waltham, MA. [cited 2017 Nov 02]. Available from: https://www.uptodate.com/contents/susceptibility-to-infections-in-persons-with-diabetes-mellitus.

157. Hogan PG, Rodriguez M, Spenner AM, Brenneisen JM, Boyle MG, Sullivan ML, et al. Impact of systemic antibiotics on *Staphylococcus aureus* colonization and recurrent skin infection. Clin Infect Dis. 2018;66:191-7. doi:10.1093/cid/cix754.

158. Feingold KR. Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. J Lipid Res. 2007;48:2531-46. doi:10.1194/jlr.R700013-JLR200.

159. Majumdar SR, McAlister FA, Eurich DT, Padwal RS, Marrie TJ. Statins and outcomes in patients admitted to hospital with community acquired pneumonia: population based prospective cohort study. BMJ. 2006;333:999. doi:10.1136/bmj.38992.565972.7C.

160. Ko HHT, Lareu RR, Dix BR, Hughes JD, Parsons RW. A sequence symmetry analysis of the interrelationships between statins, diabetes and skin infections. Br J Clin Pharmacol. 2019;85:2559-67. doi:10.1111/bcp.14077.

161. Hallas J, Pottegard A. Use of self-controlled designs in pharmacoepidemiology. J Intern Med. 2014;275:581-9. doi:10.1111/joim.12186.

162. Brookhart MA, Patrick AR, Dormuth C, Avorn J, Shrank W, Cadarette SM, et al. Adherence to lipid-lowering therapy and the use of preventive health services: an investigation of the healthy user effect. Am J Epidemiol. 2007;166:348-54. doi:10.1093/aje/kwm070.

163. Ben-Shlomo Y, Kuh D. A life course approach to chronic disease epidemiology: conceptual models, empirical challenges and interdisciplinary perspectives. Int J Epidemiol. 2002;31:285-93.

164. Hallas J. Evidence of depression provoked by cardiovascular medication: a prescription sequence symmetry analysis. Epidemiology. 1996;7:478-84.

165. Tsiropoulos I, Andersen M, Hallas J. Adverse events with use of antiepileptic drugs: a prescription and event symmetry analysis. Pharmacoepidemiol Drug Saf. 2009;18:483-91. doi:10.1002/pds.1736.

166. Australian Government Department of Veterans' Affairs. Treatment population statistics. Quarterly report - December 2012. Canberra, Australia 2012.

167. WHO Collaborating Centre for Drug Statistics Methodology. ATC/DDD Index 2018. Norway: Norwegian Institute of Public Health; 2018 [cited 2018 Mar 20]. Available from: https://www.whocc.no/atc_ddd_index/.

168. Nelson P. Socio-economic indexes for 2009 electoral divisions: 2006 Census, Research Paper No. 1 2010–11. Department of Parliamentary Services, Parliamentary Library, Canberra, 2010.

169. Tleyjeh IM, Kashour T, Hakim FA, Zimmerman VA, Erwin PJ, Sutton AJ, et al. Statins for the prevention and treatment of infections: a systematic review and meta-analysis. Arch Intern Med. 2009;169:1658-67. doi:10.1001/archinternmed.2009.286.

170. Hauer-Jensen M, Fort C, Mehta JL, Fink LM. Influence of statins on postoperative wound complications after inguinal or ventral herniorrhaphy. Hernia. 2006;10:48-52. doi:10.1007/s10029-005-0030-x.

171. van den Hoek HL, Bos WJW, de Boer A, van de Garde EMW. Statins and prevention of infections: systematic review and meta-analysis of data from large randomised placebo controlled trials. BMJ. 2011;343:d7281. doi:10.1136/bmj.d7281.

172. Brault M, Ray J, Gomez Y-H, Mantzoros CS, Daskalopoulou SS. Statin treatment and new-onset diabetes: A review of proposed mechanisms. Metabolism. 2014;63:735-45. doi:http://dx.doi.org/10.1016/j.metabol.2014.02.014.

173. Carey IM, Critchley JA, DeWilde S, Harris T, Hosking FJ, Cook DG. Risk of infection in type 1 and type 2 diabetes compared with the general population: a matched cohort study. Diabetes Care. 2018;41:513-21. doi:10.2337/dc17-2131.

174. Pouwels KB, Widyakusuma NN, Bos JH, Hak E. Association between statins and infections among patients with diabetes: a cohort and prescription sequence symmetry analysis. Pharmacoepidemiol Drug Saf. 2016;25:1124-30. doi:10.1002/pds.4052.

175. WHO Collaborating Centre for Drug Statistics Methodology. ATC/DDD Index: J - Antiinfectives for systemic use. Norway: Norwegian Institute of Public Health; 2018 [cited 2019 Jan 28]. Available from: https://www.whocc.no/atc_ddd_index/?code=J01C.

176. Liappis AP, Kan VL, Rochester CG, Simon GL. The effect of statins on mortality in patients with bacteremia. Clin Infect Dis. 2001;33:1352-7. doi:10.1086/323334.

177. Hargreaves IP, Duncan AJ, Heales SJ, Land JM. The effect of HMG-CoA reductase inhibitors on coenzyme Q10: possible biochemical/clinical implications. Drug Saf. 2005;28:659-76.

178. Pharmacometabolomic signature links simvastatin therapy and insulin resistance: an addition to the topical collection “Recent advances in pharmacometabolomics: Enabling tools for precision medicine”. Metabolomics. 2017;13:57. doi:10.1007/s11306-017-1190-2.

179. Cui JY, Zhou RR, Han S, Wang TS, Wang LQ, Xie XH. Statin therapy on glycemic control in type 2 diabetic patients: A network meta-analysis. J Clin Pharm Ther. 2018. doi:10.1111/jcpt.12690.

180. Jones ML, Martoni CJ, Ganopolsky JG, Labbe A, Prakash S. The human microbiome and bile acid metabolism: dysbiosis, dysmetabolism, disease and intervention. Expert Opin Biol Ther. 2014;14:467-82. doi:10.1517/14712598.2014.880420.

181. Mezza T, Muscogiuri G, Sorice GP, Prioletta A, Salomone E, Pontecorvi A, et al. Vitamin D deficiency: a new risk factor for type 2 diabetes? Ann Nutr Metab. 2012;61:337-48. doi:10.1159/000342771.

182. Yavuz B, Ertugrul DT. Statins and vitamin D: a hot topic that will be discussed for a long time. Dermatoendocrinol. 2012;4:8-9. doi:10.4161/derm.20188.

183. Glossmann HH, Blumthaler M. Does rosuvastatin increase serum levels of 25-hydroxy-vitamin D? Dermatoendocrinol. 2012;4:2-7. doi:10.4161/derm.18681.

184. Iruretagoyena M, Hirigoyen D, Naves R, Burgos PI. Immune response modulation by vitamin D: role in systemic lupus erythematosus. Front Immunol. 2015;6:Article 513. doi:10.3389/fimmu.2015.00513.

185. Nurieva RI, Chung Y. Understanding the development and function of T follicular helper cells. Cell Mol Immunol. 2010;7:190-7. doi:10.1038/cmi.2010.24.

186. Forero-Pena DA, Gutierrez FR. Statins as modulators of regulatory T-cell biology. Mediators Inflamm. 2013;2013:167086. doi:10.1155/2013/167086.

187. Mausner-Fainberg K, Luboshits G, Mor A, Maysel-Auslender S, Rubinstein A, Keren G, et al. The effect of HMG-CoA reductase inhibitors on naturally occurring CD4+CD25+ T cells. Atherosclerosis. 2008;197:829-39. doi:10.1016/j.atherosclerosis.2007.07.031.

188. Pasparakis M, Haase I, Nestle FO. Mechanisms regulating skin immunity and inflammation. Nat Rev Immunol. 2014;14:289-301. doi:10.1038/nri3646.

189. Feingold KR, Man MQ, Menon GK, Cho SS, Brown BE, Elias PM. Cholesterol synthesis is required for cutaneous barrier function in mice. J Clin Invest. 1990;86:1738-45. doi:10.1172/JCI114899.

190. Ravnskov U. High cholesterol may protect against infections and atherosclerosis. QJM. 2003;96:927-34. doi:10.1093/qjmed/hcg150.

191. Grundy SM, Stone NJ, Bailey AL, Beam C, Birtcher KK, Blumenthal RS, et al. 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol. Circulation. 2018;0:CIR.0000000000000625. doi:10.1161/CIR.0000000000000625.

192. Preiss D, Seshasai SR, Welsh P, Murphy SA, Ho JE, Waters DD, et al. Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy: a meta-analysis. JAMA. 2011;305:2556-64. doi:10.1001/jama.2011.860.

193. Erqou S, Lee CC, Adler AI. Statins and glycaemic control in individuals with diabetes: a systematic review and meta-analysis. Diabetologia. 2014;57:2444-52. doi:10.1007/s00125-014-3374-x.

194. Dujovne CA, Williams CD, Ito MK. What combination therapy with a statin, if any, would you recommend? Curr Atheroscler Rep. 2011;13:12-22. doi:10.1007/s11883-010-0150-3.

195. Marazzi G, Pelliccia F, Campolongo G, Cacciotti L, Massaro R, Poggi S, et al. Greater cardiovascular risk reduction with once-daily fixed combination of three

antihypertensive agents and statin versus free-drug combination: The ALL-IN-ONE trial. Int J Cardiol. 2016;222:885-7. doi:10.1016/j.ijcard.2016.07.163.

196. Herrick TM, Million RP. Tapping the potential of fixed-dose combinations. Nat Rev Drug Discov. 2007;6:513-4. doi:10.1038/nrd2334.

197. Hennekens CH. Fixed-dose combination therapy with statins: strengths, limitations, and clinical and regulatory considerations. Am J Cardiovasc Drugs. 2008;8:155-60. doi:10.2165/00129784-200808030-00002.

198. Kim TM, Kim H, Jeong YJ, Baik SJ, Yang SJ, Lee SH, et al. The differences in the incidence of diabetes mellitus and prediabetes according to the type of HMG-CoA reductase inhibitors prescribed in Korean patients. Pharmacoepidemiol Drug Saf. 2017;26:1156-63. doi:10.1002/pds.4237.

199. Gumprecht J, Gosho M, Budinski D, Hounslow N. Comparative long-term efficacy and tolerability of pitavastatin 4 mg and atorvastatin 20-40 mg in patients with type 2 diabetes mellitus and combined (mixed) dyslipidaemia. Diabetes Obes Metab. 2011;13:1047-55. doi:10.1111/j.1463-1326.2011.01477.x.

200. Park J-B, Jung J-H, Yoon YE, Kim H-L, Lee S-P, Kim H-K, et al. Long-term Effects of high-doSe pitavaStatin on Diabetogenicity in comparison with atorvastatin in patients with Metabolic syndrome (LESS-DM): study protocol for a randomized controlled trial. Trials. 2017;18:501. doi:10.1186/s13063-017-2229-4.

201. Eisenstein BI. Treatment challenges in the management of complicated skin and soft-tissue infections. Clin Microbiol Infect. 2008;14:17-25. doi:10.1111/j.1469-0691.2008.01922.x.

202. US Preventive Services Task Force. Statin use for the primary prevention of cardiovascular disease in adults: US Preventive Services Task Force recommendation statement. JAMA. 2016;316:1997-2007. doi:10.1001/jama.2016.15450.

203. Kuo C-L, Duan Y, Grady J. Unconditional or conditional logistic regression model for age-matched case–control data? Front Public Health. 2018;6:57.

204. Needham DM, Scales DC, Laupacis A, Pronovost PJ. A systematic review of the Charlson comorbidity index using Canadian administrative databases: a perspective on risk adjustment in critical care research. J Crit Care. 2005;20:12-9. doi:10.1016/j.jcrc.2004.09.007.

205. Lesens O, Methlin C, Hansmann Y, Remy V, Martinot M, Bergin C, et al. Role of comorbidity in mortality related to *Staphylococcus aureus* bacteremia: a prospective study using the Charlson weighted index of comorbidity. Infect Control Hosp Epidemiol. 2003;24:890-6. doi:10.1086/502156.

206. Armstrong DG, Meyr AJ. Risk factors for impaired wound healing and wound complications. 2018. In: UpToDate [Internet]. UpToDate, Waltham, MA. [cited 2018 May 24]. Available from: https://www.uptodate.com/contents/risk-factors-for-impaired-wound-healing-and-wound-complications.

207. Sabroe I, Postma D, Heijink I, Dockrell DH. The yin and the yang of immunosuppression with inhaled corticosteroids. Thorax. 2013;68:1085. doi:10.1136/thoraxjnl-2013-203773.

208. Rubio GA, Mundra LS, Thaller SR. Association of autoimmune connective tissue disease with abdominoplasty outcomes: a nationwide analysis of outcomes. JAMA Surgery. 2018;153:186-8. doi:10.1001/jamasurg.2017.3796.

209. Hennekens CH. Overview of primary prevention of coronary heart disease and stroke. 2018. In: UpToDate [Internet]. UpToDate, Waltham, MA. [cited 2018 May 24]. Available from: https://www.uptodate.com/contents/overview-of-primary-prevention-of-coronary-heart-disease-and-stroke#!

210. Hennekens CH, Lopez-Sendon J. Prevention of cardiovascular disease events in those with established disease or at high risk. 2018. In: UpToDate [Internet]. UpToDate, Waltham, MA. [cited 2018 May 24]. Available from: https://www.uptodate.com/contents/prevention-of-cardiovascular-disease-events-in-those-with-established-disease-or-at-high-risk.

211. Broughton T, Sington J, Beales IL. Statin use is associated with a reduced incidence of colorectal cancer: a colonoscopy-controlled case-control study. BMC Gastroenterol. 2012;12:36. doi:10.1186/1471-230X-12-36.

212. Dean AG, Sullivan KM. Open Source Statistics for Public Health. Atlanta, GA, USA2013 [cited 17 May 2018]. Available from: http://www.openepi.com/SampleSize/SSCC.htm.

213. Rahman M, Sakamoto J, Fukui T. Conditional versus unconditional logistic regression in the medical literature. J Clin Epidemiol. 2003;56:101-2. doi:10.1016/S0895-4356(02)00507-3.

214. Kouroumichakis I, Papanas N, Proikaki S, Zarogoulidis P, Maltezos E. Statins in prevention and treatment of severe sepsis and septic shock. Eur J Intern Med. 2011;22:125-33. doi:10.1016/j.ejim.2010.12.004.

215. Dobesh PP, Olsen KM. Statins role in the prevention and treatment of sepsis. Pharmacol Res. 2014;88:31-40. doi:10.1016/j.phrs.2014.04.010.

216. Tralhao AF, Ces de Souza-Dantas V, Salluh JI, Povoa PM. Impact of statins in outcomes of septic patients: a systematic review. Postgrad Med. 2014;126:45-58. doi:10.3810/pgm.2014.11.2832.

217. McCulloch DK, Robertson RP. Risk factors for type 2 diabetes mellitus. 2018. In: UpToDate [Internet]. UpToDate, Waltham, MA. [cited 2018 May 24]. Available from: https://www.uptodate.com/contents/risk-factors-for-type-2-diabetes-mellitus.

218. Kaspersen KA, Pedersen OB, Petersen MS, Hjalgrim H, Rostgaard K, Moller BK, et al. Obesity and risk of infection: results from the Danish Blood Donor Study. Epidemiology. 2015;26:580-9. doi:10.1097/EDE.0000000000000301.

219. Langley G, Hao Y, Pondo T, Miller L, Petit S, Thomas A, et al. The impact of obesity and diabetes on the risk of disease and death due to invasive Group A Streptococcus infections in adults. Clin Infect Dis. 2016;62:845-52. doi:10.1093/cid/civ1032.

220. Klop B, Elte JWF, Castro Cabezas M. Dyslipidemia in obesity: mechanisms and potential targets. Nutrients. 2013;5:1218-40. doi:10.3390/nu5041218.

221. Chang Y, Robidoux J. Dyslipidemia management update. Curr Opin Pharmacol. 2017;33:47-55. doi:10.1016/j.coph.2017.04.005.

222. Jensen MD, Ryan DH, Apovian CM, Ard JD, Comuzzie AG, Donato KA, et al. 2013 AHA/ACC/TOS Guideline for the management of overweight and obesity in adults. J Am Coll Clin Pharm. 2014;63:2985-3023. doi:10.1016/j.jacc.2013.11.004.

223. Apovian CM, Aronne LJ, Bessesen DH, McDonnell ME, Murad MH, Pagotto U, et al. Pharmacological management of obesity: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2015;100:342-62. doi:10.1210/jc.2014-3415.

224. Perreault L. Obesity in adults: Drug therapy. 2018. In: UpToDate [Internet]. UpToDate, Waltham, MA. [cited 2018 May 24]. Available from: https://www.uptodate.com/contents/obesity-in-adults-drug-therapy#!

225. Mansi I, Frei CR, Wang CP, Mortensen EM. Statins and new-onset diabetes mellitus and diabetic complications: A retrospective cohort study of US healthy adults. J Gen Intern Med. 2015;30:1599-610. doi:10.1007/s11606-015-3335-1.

226. Sugiyama T, Tsugawa Y, Tseng CH, Kobayashi Y, Shapiro MF. Different time trends of caloric and fat intake between statin users and nonusers among US adults: gluttony in the time of statins? JAMA Intern Med. 2014;174:1038-45. doi:10.1001/jamainternmed.2014.1927.

227. Yoon D, Sheen SS, Lee S, Choi YJ, Park RW, Lim HS. Statins and risk for new-onset diabetes mellitus: A real-world cohort study using a clinical research database. Medicine (Baltimore). 2016;95:e5429. doi:10.1097/MD.0000000000005429.

228. Carter AA, Gomes T, Camacho X, Juurlink DN, Shah BR, Mamdani MM. Risk of incident diabetes among patients treated with statins: population based study. BMJ. 2013;346:f2610. doi:10.1136/bmj.f2610.

229. Therapeutic Guidelines Ltd. Therapeutic guidelines: antibiotic. 12th ed. Melbourne (VIC): Therapeutic Guidelines Ltd.; 2003.

230. eTG complete [Internet]. Cellulitis and erysipelas: Melbourne (VIC): Therapeutic Guidelines Ltd.; 2019 [cited 13 Aug 2019]. Available from: https://tgldcdp-tg-org-au.smhslibresources.health.wa.gov.au/viewTopic?topicfile=cellulitis-erysipelas&guidelineName=Antibiotic#toc_d1e339.

231. Australian Government Department of Health. Statins. [Internet]. Canberra, Australia: Therapeutic Goods Administration; 2012 [cited 2018 Jun 08]. Available from: https://www.tga.gov.au/alert/statins.

232. Arnaboldi L, Corsini A. Could changes in adiponectin drive the effect of statins on the risk of new-onset diabetes? The case of pitavastatin. Atheroscler Suppl. 2015;16:1-27. doi:10.1016/S1567-5688(14)70002-9.

233. Luo Y, Liu M. Adiponectin: a versatile player of innate immunity. J Mol Cell Biol. 2016;8:120-8. doi:10.1093/jmcb/mjw012.

234. Shibata S, Tada Y, Asano Y, Hau CS, Kato T, Saeki H, et al. Adiponectin regulates cutaneous wound healing by promoting keratinocyte proliferation and migration via the ERK signaling pathway. J Immunol. 2012;189:3231-41. doi:10.4049/jimmunol.1101739.

235. Nigro E, Scudiero O, Monaco ML, Palmieri A, Mazzarella G, Costagliola C, et al. New insight into adiponectin role in obesity and obesity-related diseases. Biomed Res Int. 2014:658913. doi:10.1155/2014/658913.

236. Li S, Shin H, Ding EL, van Dam RM. Adiponectin levels and risk of type 2 diabetes: A systematic review and meta-analysis. JAMA. 2009;302:179-88. doi:10.1001/jama.2009.976.

237. Casula M, Mozzanica F, Scotti L, Tragni E, Pirillo A, Corrao G, et al. Statin use and risk of new-onset diabetes: A meta-analysis of observational studies. Nutr Metab Cardiovasc Dis. 2017;27:396-406. doi:10.1016/j.numecd.2017.03.001.

238. Ruela A, Perissinato A, Esselin de Sousa Lino M, Silva Mudrik P, Pereira G. Evaluation of skin absorption of drugs from topical and transdermal formulations. Braz J Pharm Sci. 2016;52:527-44. doi:10.1590/s1984-82502016000300018.

239. Trommer H. Overcoming the stratum corneum: the modulation of skin penetration. Skin Pharmacol Physiol. 2006;19:106-21. doi:10.1159/000091978.

240. Mortensen M, Nordestgaard B. Comparison of five major guidelines for statin use in primary prevention in a contemporary general population. Ann Intern Med. 2018;168:85-92. doi:10.7326/M17-0681.

241. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555:623-8. doi:10.1038/nature25979.

242. Burcelin R, Serino M, Chabo C, Blasco-Baque V, Amar J. Gut microbiota and diabetes: from pathogenesis to therapeutic perspective. Acta Diabetol. 2011;48:257-73. doi:10.1007/s00592-011-0333-6.

243. Harris VC, Haak BW, Boele van Hensbroek M, Wiersinga WJ. The intestinal microbiome in infectious diseases: the clinical relevance of a rapidly emerging field. Open Forum Infect Dis. 2017;4:ofx144. doi:10.1093/ofid/ofx144.

244. Poutahidis T, Kearney SM, Levkovich T, Qi P, Varian BJ, Lakritz JR, et al. Microbial symbionts accelerate wound healing via the neuropeptide hormone oxytocin. PLoS One. 2013;8:e78898. doi:10.1371/journal.pone.0078898.

245. Shehu AI, Li G, Xie W, Ma X. The pregnane X receptor in tuberculosis therapeutics. Expert Opin Drug Metab Toxicol. 2016;12:21-30. doi:10.1517/17425255.2016.1121381.

246. Dookie N, Rambaran S, Padayatchi N, Mahomed S, Naidoo K. Evolution of drug resistance in *Mycobacterium tuberculosis*: a review on the molecular determinants of resistance and implications for personalized care. J Antimicrob Chemother. 2018;73:1138-51. doi:10.1093/jac/dkx506.

247. Bhagyaraj E, Tiwari D, Ahuja N, Nanduri R, Saini A, Kalra R, et al. A human xenobiotic nuclear receptor contributes to nonresponsiveness of *Mycobacterium tuberculosis* to the antituberculosis drug rifampicin. J Biol Chem. 2018;293:3747-57. doi:10.1074/jbc.M117.818377.

248. Hukkanen J, Hakkola J, Rysa J. Pregnane X receptor (PXR) - a contributor to the diabetes epidemic? Drug Metabol Drug Interact. 2014;29:3-15. doi:10.1515/dmdi-2013-0036.

249. Krausova L, Stejskalova L, Wang H, Vrzal R, Dvorak Z, Mani S, et al. Metformin suppresses pregnane X receptor (PXR)-regulated transactivation of CYP3A4 gene. Biochem Pharmacol. 2011;82:1771-80. doi:10.1016/j.bcp.2011.08.023.

250. Zhao P, Sui BD, Liu N, Lv YJ, Zheng CX, Lu YB, et al. Anti-aging pharmacology in cutaneous wound healing: effects of metformin, resveratrol, and rapamycin by local application. Aging Cell. 2017;16:1083-93. doi:10.1111/acel.12635.

APPENDICES

PRISMA 2009 Checklist for "Statins: Antimicrobial resistance breakers or makers?"

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Lines 15-18, 103-16 (Multifaceted narrative review reflected in paper, not in title)
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Lines 11-47 (Narrative review, no registration number)
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Lines 49-66
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Lines 103-106
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	Narrative review, no registration number
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Lines 121-144
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Lines 109-119
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Lines 109-119 and Figure 1
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Lines 121-144
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Lines 109-155 and Figure 1, data extraction done by first author (HK)
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Lines 146-155, Tables 1 and 2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Lines 146-155, 203-233, Tables 1 and 2
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	Minimum inhibitory concentrations of respective statins against various bacterial strains
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	Lines 146-200

PRISMA 2009 Checklist for "Statins: Antimicrobial resistance breakers or makers?"

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Lines 203-233
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	Lines 235-358, 484-524
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Lines 109-155 and Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Lines 146-155, Tables 1 and 2
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	Lines 203-233
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	(a) Tables 1 and 2 (b) Not applicable
Synthesis of results	21	Present the main results of the review. If meta-analyses are done, include for each, confidence intervals and measures of consistency.	Lines 157-200, Tables 1 and 2 (no meta-analysis done)
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	Lines 203-233
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	Lines 235-358, 484-524
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	Lines 235-358, 484-524
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	Study level (lines 203-233); review level (lines 116-119, literature search by only one author)
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Line 527-556
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Lines 575-578

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Appendix 2: Compiled antimicrobial susceptibility results of statins against various Gram-positive bacteria reported in literature

Compiled antimicrobial susceptibility results of statins against various Gram-positive bacteria reported in literature.[a]

Bacteria type and strain[b]	Solvent/Broth[c]	Statin (MIC in µg/mL)[d]							Reference[e]
		ATV	FLV	LVS	PTV	PRV	RSV	SMV	
Bacillus species									
Isolates	Methanol 1:2 dilution (range from 50% to 0.78%)	43.75 ± 17.12	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	(Radwan & Ezzat 2012)
Bacillus anthracis									
AMES35, UM23	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	16	(Thangamani et al. 2015)
Enterococcus faecalis									
Unknown strain	Ethanol 1%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Quivey 2014)
Enterococcus faecalis **(Vancomycin-resistant)**									
ATCC 51299	DMSO Unknown %	166.67 ± 72.16	Not tested	Not tested	Not tested	Not tested	500 ± 0.00	104.17 ± 36.08	(Masadeh et al. 2012)
ATCC 51299	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
ATCC 51299	Ethanol 6.25%	250	Not tested	Not tested	Not tested	Not tested	100	Not tested	(Welsh et al. 2009)
SF24413, SF28073	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Isolates	DMSO Unknown %	216.67 ± 32.27	Not tested	Not tested	Not tested	Not tested	500 ± 0.00	291.67 ± 39.53	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Enterococcus faecalis **(Vancomycin-sensitive)**									
ATCC 7080, ATCC 14506	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
ATCC 19433	DMSO Unknown %	83.33 ± 36.08	Not tested	Not tested	Not tested	Not tested	333.33 ± 144.33	52.08 ± 18.04	(Masadeh et al. 2012)
ATCC 29212	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Coban et al. 2010)
ATCC 29212	Ethanol 6.25%	250	Not tested	Not tested	Not tested	Not tested	100	Not tested	(Welsh et al. 2009)
ATCC 29212	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	>250	(Graziano et al. 2015)
ATCC 49532, ATCC 49533, HH22, MMH594, SF24397	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Isolates	DMSO Unknown %	95.83 ± 22.09	Not tested	Not tested	Not tested	Not tested	333.33 ± 0.00	291.67 ± 39.53	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)

(continued on next page)

Compiled antimicrobial susceptibility results of statins against various Gram-positive bacteria reported in literature.[a] ***(continued)***

		Statin (MIC in μg/mL)[d]							
Bacteria type and strain[b]	**Solvent/Broth**[c]	**ATV**	**FLV**	**LVS**	**PTV**	**PRV**	**RSV**	**SMV**	**Reference**[e]
Enterococcus faecium									
Unknown strain	Ethanol 1%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Quivey 2014)
***Enterococcus faecium* (Vancomycin-resistant)**									
ATCC 700221, E0120, ERV102	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
***Enterococcus faecium* (Vancomycin-sensitive)**									
ATCC 6569, E1162	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Lactobacillus casei									
Unknown strain	Not specified	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	7.8	(Ting et al. 2016)
Listeria monocytogenes									
ATCC 13932, ATCC 19111, ATCC 19112, ATCC 19114, F4244, J0161	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Staphylococci (Methicillin-resistant coagulase negative, MRCoNS)									
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Staphylococcus aureus									
Unknown strain	Ethanol 1%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Quivey 2014)
***Staphylococcus aureus* (Methicillin-resistant, MRSA)**									
ATCC 14458, ATCC 33591, ATCC 43300	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	31.25	(Graziano et al. 2015)
ATCC 43300	DMSO Unknown %	83.33 ± 36.08	Not tested	Not tested	Not tested	Not tested	500 ± 0.00	166.67 ± 72.16	(Masadeh et al. 2012)
ATCC 43300	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
ATCC 43300	Unknown solvent and %	>1024	>1024	>1024	>1024	>1024	>1024	32	(Thangamani et al. 2015)
ATCC 49476	Ethanol 6.25%	250	Not tested	Not tested	Not tested	Not tested	100	Not tested	(Welsh et al. 2009)
ATCC BAA-44, NRS70, NRS71, NRS108, NRS119, NRS123	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)

(continued on next page)

Compiled antimicrobial susceptibility results of statins against various Gram-positive bacteria reported in literature.[a] *(continued)*

		Statin (MIC in μg/mL)[d]							
Bacteria type and strain[b]	**Solvent/Broth[c]**	**ATV**	**FLV**	**LVS**	**PTV**	**PRV**	**RSV**	**SMV**	**Reference[e]**
MRSA (continued)									
NRS100, NRS194	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Thangamani et al. 2015)
USA100, USA200, USA300, USA400, USA500, USA700, USA800, USA1000, USA1100	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Isolates	DMSO Unknown %	108.33 ± 27.36	Not tested	Not tested	Not tested	Not tested	500.00 ± 0.00	116.67 ± 30.19	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Isolates	Methanol 1:2 dilution (range from 50% to 0.2%)	Not tested	>200 (mean)	Not tested	Not tested	Not tested	Not tested	74.9 (mean)	(Jerwood & Cohen 2008)
Isolates	Methanol 1:2 dilution (range from 50% to 0.78%)	37.5 ± 13.98	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	(Radwan & Ezzat 2012)
***Staphylococcus aureus* (Methicillin-sensitive, MSSA)**									
ATCC 6538	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	31.25	(Graziano et al. 2015)
ATCC 6538	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
ATCC 25213	DMSO Unknown %	41.67 ± 18.04	Not tested	Not tested	Not tested	Not tested	208.33 ± 72.16	26.04 ± 9.02	(Masadeh et al. 2012)
ATCC 25923	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Coban et al. 2010)
ATCC 25923	Ethanol 6.25%	250	Not tested	Not tested	Not tested	Not tested	100	Not tested	(Welsh et al. 2009)
ATCC 29213	DMSO 0.5%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	62.5	(Wang et al. 2015)
ATCC 29213	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Coban et al. 2010)
ATCC 29213	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	15.65	(Graziano et al. 2015)
ATCC 29213	Various solvents and %	>250 (Ethanol 5%)	500	>500 (DMSO 5%)	Not tested	>500	>500	31 (Methanol 100%); 500 (Methanol 5%); 500 (SMV sodium)	(Matzneller et al. 2011)
RN4220, NRS72, NRS77, NRS846, NRS860	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)

(continued on next page)

Compiled antimicrobial susceptibility results of statins against various Gram-positive bacteria reported in literature.[a] ***(continued)***

		Statin (MIC in µg/mL)[d]							
Bacteria type and strain[b]	**Solvent/Broth**[c]	**ATV**	**FLV**	**LVS**	**PTV**	**PRV**	**RSV**	**SMV**	**Reference**[e]
MSSA *(continued)*									
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Isolates	DMSO Unknown %	52.08 ± 11.04	Not tested	Not tested	Not tested	Not tested	341.67 ± 20.84	60.42 ± 12.76	(Masadeh et al. 2012)
Isolates	Methanol 1:2 dilution (range from 50% to 0.2%)	Not tested	>200 (mean)	Not tested	Not tested	Not tested	Not tested	29.2 (mean)	(Jerwood & Cohen 2008)
Isolates	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	31.25	(Graziano et al. 2015)
***Staphylococcus aureus* (Vancomycin-intermediate, VISA)**									
NRS1, NRS19, NRS37	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
***Staphylococcus aureus* (Vancomycin-resistant, VRSA)**									
VRS1, VRS2, VRS3a, VRS3b, VRS4, VRS5, VRS6, VRS7, VRS8, VRS10, VRS11a, VRS11b, VRS12, VRS13	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
VRS9	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Thangamani et al. 2015)
Staphylococcus epidermidis									
ATCC 12228	DMSO Unknown %	20.83 ± 9.02	Not tested	Not tested	Not tested	Not tested	166.67 ± 72.16	26.04 ± 9.02	(Masadeh et al. 2012)
NRS101	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	32	(Thangamani et al. 2015)
Isolates	DMSO Unknown %	19.78 ± 4.94	Not tested	Not tested	Not tested	Not tested	233.33 ± 39.52	35.41 ± 4.94	(Masadeh et al. 2012)
Streptococcus anginosus									
Unknown strain	Not specified	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	7.8	(Ting et al. 2016)
Streptococcus mutans									
ATCC 25175	DMSO 1:2 dilution (range from 50% to 0.2%)	100	Not tested	Not tested	Not tested	200	100	15.6	(Alshammari 2016)
UA159	Ethanol 1%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	16	(Quivey 2014)
Unknown strain	Not specified	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	15.6	(Ting et al. 2016)

(continued on next page)

Compiled antimicrobial susceptibility results of statins against various Gram-positive bacteria reported in literature.[a] *(continued)*

		Statin (MIC in µg/mL)[d]							
Bacteria type and strain[b]	**Solvent/Broth[c]**	**ATV**	**FLV**	**LVS**	**PTV**	**PRV**	**RSV**	**SMV**	**Reference[e]**
Streptococcus pneumoniae									
51916, 70677	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Thangamani et al. 2015)
ATCC BAA-334	DMSO 2.5%	Not tested	>100	Not tested	Not tested	>100	Not tested	15.6	(Bergman et al. 2011)
Unknown ATCC strain	DMSO Unknown %	104.17 ± 36.08	Not tested	Not tested	Not tested	Not tested	333.33 ± 144.33	166.67 ± 72.16	(Masadeh et al. 2012)
Isolates	DMSO Unknown %	229.17 ± 60.38	Not tested	Not tested	Not tested	Not tested	416.67 ± 0.00	291.67 ± 39.53	(Masadeh et al. 2012)
Unknown strain	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	15	(Bjorkhem-Bergman et al. 2011)
Streptococcus pyogenes									
ATCC 19615	DMSO Unknown %	83.33 ± 36.08	Not tested	Not tested	Not tested	Not tested	166.67 ± 72.16	62.5 ± 0.00	(Masadeh et al. 2012)
Isolates	DMSO Unknown %	133.33 ± 19.76	Not tested	Not tested	Not tested	Not tested	275.00 ± 72.17	145.83 ± 32.27	(Masadeh et al. 2012)
Streptococcus salivarius									
ATCC 2593	DMSO 1:2 dilution (range from 50% to 0.2%)	100	Not tested	Not tested	Not tested	200	100	7.8	(Alshammari 2016)
Unknown strain	Not specified	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	7.8	(Ting et al. 2016)
Streptococcus sanguinis (Streptococcus sanguis)									
ATCC 10556	DMSO 1:2 dilution (range from 50% to 0.2%)	100	Not tested	Not tested	Not tested	200	100	15.6	(Alshammari 2016)
Unknown strain	Not specified	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	15.6	(Ting et al. 2016)

[a] The dilution methods for Bergman et al. (2011), Quivey (2014), Welsh, Kruger & Faoagali (2009), and Ting, Whitaker & Albandar (2016) were described in the respective studies. All other studies were tested according to the broth microdilution method stipulated by the Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards (NCCLS).

[b] ATCC, American Type Culture Collection.

[c] All studies were tested with Mueller Hinton broth unless specified. Solvent types and solvent concentrations used for water insoluble statins (ATV, LVS, PTV, and SMV) were listed as reported in the various references. DMSO, dimethyl sulfoxide.

[d] ATV, atorvastatin; FLV, fluvastatin; LVS, lovastatin; MIC, minimum inhibitory concentration; PRV, pravastatin; PTV, pitavastatin; RSV, rosuvastatin; SMV, simvastatin.

[e] Corresponding thesis reference numbers: Alshammari 2016 [53]; Bergman et al. 2011 [58]; Bjorkhem-Bergman et al. 2011 [54]; Coban et al. 2010 [55]; Graziano et al. 2015 [60]; Jerwood & Cohen 2008 [42]; Masadeh et al. 2012 [44]; Matzneller, Manafi & Zeitlinger 2011 [61]; Quivey 2014 [56]; Radwan & Ezzat 2012 [62]; Thangamani et al. 2015 [43]; Ting, Whitaker & Albandar 2016 [57]; Wang et al. 2016 [64]; Welsh, Kruger & Faoagali 2009 [65].

Appendix 3: Compiled antimicrobial susceptibility results of statins against various Gram-negative bacteria reported in literature

Compiled antimicrobial susceptibility results of statins against various Gram-negative bacteria reported in literature.[a]

Bacteria type and strain[b]	Solvent/Broth[c]	Statin (MIC in µg/mL)[d]							Reference[e]
		ATV	FLV	LVS	PTV	PRV	RSV	SMV	
Acinetobacter baumannii									
ATCC 17978	DMSO Unknown %	15.62 ± 0.00	Not tested	Not tested	Not tested	Not tested	333.33 ± 144.33	104.17 ± 36.08	(Masadeh et al. 2012)
ATCC BAA747, ATCC BAA1605, ATCC BAA19606	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
Isolates	DMSO Unknown %	21.87 ± 4.94	Not tested	Not tested	Not tested	Not tested	300.00 ± 79.05	32.29 ± 6.38	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Aggregatibacter actinomycetemcomitans									
Unknown ATCC strain	DMSO 1% stock, Brain heart infusion broth	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	<1	(Emani et al. 2014)
Unknown strain	Not specified	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	3.95	(Ting et al. 2016)
Citrobacter freundii									
ATCC 8090	DMSO Unknown %	83.33 ± 36.08	Not tested	Not tested	Not tested	Not tested	166.67 ± 72.16	52.08 ± 18.04	(Masadeh et al. 2012)
Isolates	DMSO Unknown %	108.33 ± 27.36	Not tested	Not tested	Not tested	Not tested	333.33 ± 79.06	133.33 ± 39.58	(Masadeh et al. 2012)
Enterobacter aerogenes									
ATCC 29751	DMSO Unknown %	15.62 ± 0.00	Not tested	Not tested	Not tested	Not tested	104.17 ± 36.08	26.04 ± 9.02	(Masadeh et al. 2012)
Isolates	DMSO Unknown %	19.78 ± 4.94	Not tested	Not tested	Not tested	Not tested	183.33 ± 0.00	33.33 ± 4.94	(Masadeh et al. 2012)
Enterobacter cloacae									
ATCC 13047	DMSO Unknown %	41.67 ± 18.04	Not tested	Not tested	Not tested	Not tested	166.67 ± 72.16	62.5 ± 0.00	(Masadeh et al. 2012)
Isolates	DMSO Unknown %	113.54 ± 27.06	Not tested	Not tested	Not tested	Not tested	316.67 ± 64.55	143.75 ± 36.97	(Masadeh et al. 2012)
Escherichia coli									
1411, SM1411Δ *acrAB*	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
ATCC 10536, ATCC 25922	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	>250	(Graziano et al. 2015)
ATCC 25922	Various solvents and %	>250 (Ethanol 5%)	500	>500 (DMSO 5%)	Not tested	>500	>500	>500 (Methanol 100% and 5%)	(Matzneller et al. 2011)
ATCC 25922	Ethanol 6.25%	250	Not tested	Not tested	Not tested	Not tested	100	Not tested	(Welsh et al. 2009)
ATCC 35218	DMSO Unknown %	26.04 ± 9.02	Not tested	Not tested	Not tested	Not tested	104.17 ± 36.08	52.08 ± 18.04	(Masadeh et al. 2012)

(continued on next page)

Compiled antimicrobial susceptibility results of statins against various Gram-negative bacteria reported in literature.[a] *(continued)*

		Statin (MIC in µg/mL)[d]							
Bacteria type and strain[b]	**Solvent/Broth[c]**	**ATV**	**FLV**	**LVS**	**PTV**	**PRV**	**RSV**	**SMV**	**Reference[e]**
Escherichia coli (continued)									
ATCC 35218	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Isolates	DMSO Unknown %	100.00 ± 33.75	Not tested	Not tested	Not tested	Not tested	125.00 ± 16.14	112.5 ± 30.19	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Isolates	Methanol 1:2 dilution (range from 50% to 0.78%)	75 ± 27.95	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	(Radwan & Ezzat 2012)
***Escherichia coli* O157:H7**									
ATCC 35150, ATCC 700728	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
Haemophilus influenzae									
ATCC 29247	DMSO Unknown %	83.33 ± 36.08	Not tested	Not tested	Not tested	Not tested	166.67 ± 72.16	52.08 ± 18.04	(Masadeh et al. 2012)
Isolates	DMSO Unknown %	104.17 ± 36.08	Not tested	Not tested	Not tested	Not tested	366.67 ± 0.00	145.83 ± 32.27	(Masadeh et al. 2012)
Isolates	DMSO 2.5%	Not tested	>100	Not tested	Not tested	>100	Not tested	>250	(Bergman et al. 2011)
Klebsiella species									
Not specified	Ethanol 1%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	64	(Quivey 2014)
Klebsiella pneumoniae									
ATCC 13883	DMSO Unknown %	166.67 ± 72.16	Not tested	Not tested	Not tested	Not tested	333.33± 144.33	166.67 ± 72.16	(Masadeh et al. 2012)
ATCC 700603	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
ATCC BAA-1705, ATCC BAA-2146	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
Isolates	DMSO Unknown %	216.67 ± 51.03	Not tested	Not tested	Not tested	Not tested	258.33 ± 64.55	241.67 ± 60.38	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Moraxella catarrhalis									
Isolates	DMSO 2.5%	Not tested	>100	Not tested	Not tested	>100	Not tested	15.6	(Bergman et al. 2011)
Porphyromonas gingivalis									
ATCC 33277	DMSO 1% stock, Brain heart infusion broth	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	2	(Emani et al. 2014)
Proteus mirabilis									
ATCC 12459	DMSO Unknown %	62.5 ± 0.00	Not tested	Not tested	Not tested	Not tested	250 ± 0.00	166.67 ± 72.16	(Masadeh et al. 2012)

(continued on next page)

Compiled antimicrobial susceptibility results of statins against various Gram-negative bacteria reported in literature.[a] *(continued)*

		Statin (MIC in µg/mL)[d]							
Bacteria type and strain[b]	**Solvent/Broth[c]**	**ATV**	**FLV**	**LVS**	**PTV**	**PRV**	**RSV**	**SMV**	**Reference[e]**
Proteus mirabilis (continued)									
Isolates	DMSO Unknown %	127.08 ± 25.51	Not tested	Not tested	Not tested	Not tested	191.67 ± 32.27	158.33 ± 32.27	(Masadeh et al. 2012)
Isolates	Methanol 1:2 dilution (range from 50% to 0.78%)	125 ± 0.00	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	(Radwan & Ezzat 2012)
Pseudomonas aeruginosa									
ATCC 9027	DMSO Unknown %	83.33 ± 36.08	Not tested	Not tested	Not tested	Not tested	166.67 ± 72.16	166.67 ± 72.16	(Masadeh et al. 2012)
ATCC 9027, ATCC 9721, ATCC 10145	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
ATCC 15442	Unknown solvent and %	>1024	>1024	>1024	>1024	>1024	>1024	>1024	(Thangamani et al. 2015)
ATCC 25619	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	>250	(Graziano et al. 2015)
ATCC 25619, ATCC 27853	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
ATCC 27853	DMSO 2.5%	>250	Not tested	Not tested	Not tested	>250	Not tested	>250	(Graziano et al. 2015)
ATCC 27853	Various solvents and %	>250 (Ethanol 5%)	500	>500 (DMSO 5%)	Not tested	>500	>500	>500 (Methanol 100% and 5%)	(Matzneller et al. 2011)
ATCC 27853	Ethanol 6.25%	250	Not tested	Not tested	Not tested	Not tested	100	Not tested	(Welsh et al. 2009)
ATCC 35032, ATCC BAA-1744	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)
PAO1	DMSO 2% stock, Lysogeny Broth	625	Not tested	Not tested	Not tested	Not tested	625	Not tested	(Sarabhai et al. 2015)
Isolates	DMSO Unknown %	95.83 ± 22.09	Not tested	Not tested	Not tested	Not tested	291.67 ± 39.53	120.83 ± 32.27	(Masadeh et al. 2012)
Isolates	Unknown solvent and %	>128	Not tested	Not tested	Not tested	Not tested	Not tested	>128	(Coban et al. 2010)
Unknown strain	Ethanol 1%	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Quivey 2014)
Salmonella Typhimurium									
ATCC 700720	Unknown solvent and %	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	>256	(Thangamani et al. 2015)

[a] The dilution methods for (Bergman et al. 2011), (Quivey 2014), (Welsh et al. 2009) and (Ting et al. 2016) were described in the respective studies. All other studies were tested according to the broth microdilution method stipulated by the Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards (NCCLS).

[b] ATCC, American Type Culture Collection.

[c] All studies were tested with Mueller Hinton broth unless specified. Solvent types and solvent concentrations used for water insoluble statins (atorvastatin, lovastatin, pitavastatin and simvastatin) were listed as reported in the various references. DMSO, dimethyl sulfoxide.

[d] ATV, atorvastatin; FLV, fluvastatin; LVS, lovastatin; MIC, minimum inhibitory concentration; PRV, pravastatin; PTV, pitavastatin; RSV, rosuvastatin; SMV, simvastatin.

[e] Corresponding thesis reference numbers: Bergman et al. 2011 [58]; Coban et al. 2010 [55]; Emani, Gunjiganur & Mehta 2014 [59]; Graziano et al. 2015 [60]; Masadeh et al. 2012 [44]; Matzneller, Manafi & Zeitlinger 2011 [61]; Quivey 2014 [56]; Radwan & Ezzat 2012 [62]; Sarabhai et al. 2015 [63]; Thangamani et al. 2015 [43]; Ting, Whitaker & Albandar 2016 [57]; Welsh, Kruger & Faoagali 2009 [65].

Appendix 4: Co-authors' permission to include published paper (*PeerJ*)[47] as Chapter Two of thesis

---

Ko H, Lareu RR, Dix BR, Hughes JD. Statins: antimicrobial resistance breakers or makers? PeerJ. 2017;5:e3952.

I, Hean Teik Humphrey Ko, performed the literature and reference searches, collected the data, prepared the figures and tables, wrote the manuscript, and contributed significantly to the design, analysis, and interpretation of findings as lead author in the above peer-reviewed article published in PeerJ on 24th October 2017.

_____ _____

Hean Teik Humphrey Ko (Signature) *Date*

We, as Co-Authors, endorse that the contributions indicated above by the doctorial candidate, Hean Teik Humphrey Ko, are appropriate. We permit the contents of the above manuscript to be included in his PhD thesis for examination and to be added to Curtin University's digital institutional repository.

_____ _____

Dr Ricky R. Lareu (Signature) *Date*

_____ _____

Dr Brett R. Dix (Signature) *Date*

_____ _____

Prof Jeffery D. Hughes (Signature) *Date*

Appendix 5: License agreement for published paper (*Eur J Clin Microbiol Infect Dis*)[130] to be used in thesis for examination (embargo period till 17th May 2019)

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Jun 25, 2018

This Agreement between Mr. Humphrey Ko ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4351380600962
License date	May 17, 2018
Licensed Content Publisher	Springer Nature
Licensed Content Publication	European Journal of Clinical Microbiology and Infectious Diseases
Licensed Content Title	In vitro antibacterial effects of statins against bacterial pathogens causing skin infections
Licensed Content Author	Humphrey H. T. Ko, Ricky R. Lareu, Brett R. Dix et al
Licensed Content Date	Jan 1, 2018
Licensed Content Volume	37
Licensed Content Issue	6
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	full article/chapter
Will you be translating?	no
Circulation/distribution	501 to 1000
Author of this Springer Nature content	yes
Title	Investigating the Relationship Between Statins and Bacterial Skin Infections
Instructor name	Humphrey Ko
Institution name	Curtin University, Western Australia
Expected presentation date	Jun 2018
Order reference number	Not applicable
Requestor Location	Mr. Humphrey Ko Curtin University, Bentley, Perth,, Western Australia 6102 Australia Attn: Mr. Humphrey Ko
Billing Type	Invoice
Billing Address	Mr. Humphrey Ko Curtin University, Bentley,

Appendix — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT]** (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.0

Appendix 6: Co-authors' permission to include published paper (*European Journal of Clinical Microbiology and Infectious Diseases*)[130] as Chapter Three of thesis

---

Ko H, Lareu RR, Dix BR, Hughes JD. *In vitro* antibacterial effects of statins against bacterial pathogens causing skin infections. Eur J Clin Microbiol Infect Dis. 2018. DOI:10.1007/s10096-018-3227-5.

I, Hean Teik Humphrey Ko, performed the literature and reference searches, collected the data, prepared the figures and tables, wrote the manuscript, and contributed significantly to the design, analysis, and interpretation of findings as lead author in the above peer-reviewed article first published online in the European Journal of Clinical Microbiology & Infectious Diseases on 22nd March 2018.

_____ _____

Hean Teik Humphrey Ko (Signature) *Date*

We, as Co-Authors, endorse that the contributions indicated above by the doctorial candidate, Hean Teik Humphrey Ko, are appropriate. We permit the contents of the above manuscript to be included in his PhD thesis for examination and to be added to Curtin University's digital institutional repository.

_____ _____

Dr Ricky R. Lareu (Signature) *Date*

_____ _____

Dr Brett R. Dix (Signature) *Date*

_____ _____

Prof Jeffery D. Hughes (Signature) *Date*

Appendix 7: License agreement for published paper (*Br J Clin Pharmacol*)[160] to be used in thesis for examination (embargo period till 8th October 2020)

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Oct 18, 2019

This Agreement between Mr. Humphrey Ko ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4691910799284
License date	Oct 18, 2019
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	British Journal of Clinical Pharmacology
Licensed Content Title	A sequence symmetry analysis of the interrelationships between statins, diabetes and skin infections
Licensed Content Author	Humphrey H.T. Ko, Ricky R. Lareu, Brett R. Dix, et al
Licensed Content Date	Oct 10, 2019
Licensed Content Volume	0
Licensed Content Issue	0
Licensed Content Pages	9
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Investigating the Relationship Between Statins and Bacterial Skin Infections
Expected completion date	Oct 2019
Expected size (number of pages)	190
Requestor Location	Mr. Humphrey Ko Curtin University, Bentley, Perth,, Western Australia 6102 Australia Attn: Mr. Humphrey Ko
Publisher Tax ID	EU826007151
Total	0.00 AUD
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at

http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order,** is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner.**For STM Signatory Publishers clearing permission under the terms of the STM Permissions Guidelines only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts,** You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED

WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be

binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

Appendix 8: Co-authors' permission to include manuscript submitted for consideration of publication in the *Medical Journal of Australia* as Chapter Four of thesis (subsequently accepted for publication in the *British Journal of Clinical Pharmacology*)[160]

Ko H, Lareu RR, Dix BR, Hughes JD, Parsons R. A sequence symmetry analysis of the interrelationships between statins, diabetes, and skin infections. (Manuscript for submission to the Medical Journal of Australia)

I, Hean Teik Humphrey Ko, performed the literature and reference searches, collected the data, prepared the figures and tables, wrote the manuscript, and contributed significantly to the design, analysis, and interpretation of findings as lead author in the above manuscript, which will be submitted to the Medical Journal of Australia for peer-review.

_____ _____

Hean Teik Humphrey Ko (Signature) *Date*

We, as Co-Authors, endorse that the contributions indicated above by the doctorial candidate, Hean Teik Humphrey Ko, are appropriate. We permit the contents of the above manuscript to be included in his PhD thesis for examination and to be added to Curtin University's digital institutional repository.

_____ _____

Dr Ricky R. Lareu (Signature) *Date*

_____ _____

Dr Brett R. Dix (Signature) *Date*

_____ _____

Prof Jeffery D. Hughes (Signature) *Date*

_____ _____

Dr Richard Parsons (Signature) *Date*

Appendix 9: Ethics approval from the Australian DVA for research work in Chapter Four of thesis

Australian Government

Department of Veterans' Affairs

Reference: E014/003
Contact: Ms Megan MacDonald
Telephone: (02) 6225 4659
Facsimile: (02) 6289 6227
E-mail: ethics.committee@dva.com.au

Mr Hean Teik Humphrey Ko
School of Pharmacy
Curtin University
BENTLEY WA 6102

h.ko2@postgrad.curtin.edu.au

Dear Mr Ko

Ref: E014/003 Investigating the relationship between statins and skin infections

Thank you for submitting the above proposal for consideration by the Department of Veterans' Affairs Human Research Ethics Committee (DVA HREC).

The Committee originally considered this proposal at its meeting held on 28 February 2014 and provided its in-principle approval of this project, pending agreement that the data request be limited to de-identified data for medication history only, for veterans and war widows (excluding dependants). A decision letter advising of the Committee's decision was dispatched on 28 March 2014.

Thank you for your response letter dated 3 April 2014, advising that you agree with the conditions set by the Committee. I also note that you have confirmed that you will adhere to the Australian Privacy Principles (APPs) which came into effect from 12 March 2014.

I am pleased to advise that the Chair considered your response on 4 April 2014 and agreed that your proposal meets the requirements of the *National Statement on Ethical Conduct in Research Involving Humans*.

The Chair granted a waiver of the usual requirement for consent of the individual to use their health information in a research project, in line with the Guidelines approved under Section 95/95A of the *Privacy Act 1988*.

The Committee requires six-monthly progress reports from approved projects, until it receives a final report detailing the research outcome(s), or advice that the project has been suspended or abandoned.

It is the responsibility of the researcher to ensure that progress reports are submitted in a timely manner. Submission of progress reports can be made by e-mail to ethics.committee@dva.gov.au quoting reference number **E014/003**.

The Committee looks forward to receiving your first progress report by no later than **4 October 2014**. The report should address the questions set out in the Biannual Compliance Report template, available from the DVA HREC website at www.dva.gov.au/ethics.

Failure to comply with the above reporting requirements may result in withdrawal of DVA HREC approval.

Any variation from the agreed protocol or conditions of approval will require the Committee's separate consideration. The Committee should also receive immediate notification of any unexpected adverse event arising from the research.

The Committee reserves the right at any time to seek further information, noting this may affect the continuation of its approval.

The Committee has asked that you note that ethical approval does not guarantee access to DVA data, information or assistance. I note that you have lodged a request for data that is currently being processed by the relevant business area, who will also be notified of the DVA HREC's approval. You will be advised of the outcome of this data request in due course.

Please feel free to contact the DVA HREC Secretariat to discuss any matters relating to the above study on (02) 6225 4659 or via the Committee's email address ethics.committee@dva.gov.au.

Yours sincerely

Kyleigh Heggie

Kyleigh Heggie
Director
Research Development and Coordination

7 April 2014

Appendix 10: Calculating null-effect sequence ratio (NSR) for Chapter Four of thesis

---

The NSR is the expected sequence ratio in the absence of any causal relationship between the index and marker drugs, and it is used to adjust for incidence trend changes.[164, 165] The overall average probability (*Pa*) of an index→marker sequence may be calculated as an average for all days, weighted by the number of incident index drug users on consecutive (m) days of the study as follows:[1]

$$Pa = \frac{\sum_{m=1}^{u}\left[I_m \times \left(\sum_{n=m+1}^{m+d} M_n\right)\right]}{\sum_{m=1}^{u}\left[I_m \times \left(\sum_{n=m-d}^{m-1} M_n + \sum_{n=m+1}^{m+d} M_n\right)\right]}$$

where:

m or n = consecutive days of the study period excluding the run-in period

d = number of days for observation (window period of 91, 182, or 365 days in our study)

u = last day of the study period

I_m = number of people receiving their first index drug on the date

M_n = number of people receiving their first marker drug on the date

NSR is thus calculated as:

$$\text{NSR} = \frac{Pa}{(1 - Pa)}$$

Appendix 11: Anatomical Therapeutic Chemical codes and respective Daily Defined Dose used in Chapter Four of thesis

The Anatomical Therapeutic Chemical codes of medications and respective Daily Defined Dose as defined by the World Health Organization[167] that were used in this study included:

Drug name	Anatomical Therapeutic Chemical code	Daily Defined Dose
Statins		
Atorvastatin	C10AA05	20 mg
Fluvastatin	C10AA04	60 mg
Pravastatin	C10AA03	30 mg
Rosuvastatin	C10AA07	10 mg
Simvastatin	C10AA01	30 mg
Antidiabetic medication		
Acarbose	A10BF01	0.3 g
Exenatide	A10BX04 (before 2017)	0.286 mg (depot) 15 mcg
Glibenclamide	A10BB01	10 mg
Gliclazide	A10BB09	60 mg
Glimepiride	A10BB12	2 mg
Glipizide	A10BB07	10 mg
Insulin (human, fast-acting)	A10AB01	40 units
Insulin (beef, fast-acting)	A10AB02	40 units
Insulin (lispro)	A10AB04	40 units
Insulin (aspart)	A10AB05	40 units
Insulin (glulisine)	A10AB06	40 units
Insulin (human, intermediate-acting)	A10AC01	40 units
Insulin (beef, intermediate-acting)	A10AC02	40 units
Insulins and analogues for injection (intermediate or long-acting combined with fast-acting)	A10AD	-
Insulin (human, intermediate or long-acting combined with fast-acting)	A10AD01	40 units
Insulin (intermediate or long-acting combined with lispro)	A10AD04	40 units
Insulin (human, long-acting)	A10AE01	40 units
Insulin (glargine)	A10AE04	40 units
Insulin (detemir)	A10AE05	40 units
Linagliptin	A10BH05	5 mg
Metformin	A10BA02	2 g
Metformin and rosiglitazone	A10BD03	-
Metformin and sulfonylureas	A10BD02	-
Metformin and sitagliptin	A10BD07	-
Metformin and vildagliptin	A10BD08	-
Pioglitazone	A10BG03	30 mg
Rosiglitazone	A10BG02	6 mg

(continued on next page)

Drug name	**Anatomical Therapeutic Chemical code**	**Daily Defined Dose**
Antidiabetic medication *(continued)*		
Saxagliptin	A10BH03	5 mg
Sitagliptin	A10BH01	0.1 g
Tolbutamide	A10BB03	1.5 g
Vildagliptin	A10BH02	0.1 g
Antistaphylococcal antibiotics		
Dicloxacillin	J01CF01	2 g
Flucloxacillin	J01CF05	2 g

Appendix 12: Admission diagnoses for control group in Chapter Five

Admission diagnosis	Number of patients	Admission diagnosis	Number of patients
Pain (chest)	16	Drug overdose	1
Community acquired pneumonia	11	Dysphagia	1
Chronic obstructive pulmonary disease exacerbation	9	Endocarditis	1
Urinary tract infection	6	Endothelial ablation	1
Bronchitis	5	Fever	1
Congestive heart failure	5	Fracture (clavicle)	1
Pain (abdominal)	5	Fracture (hip)	1
Senile cataract	5	Gastroscopy (polyps)	1
Stroke	5	Haematuria	1
Atrial fibrillation	4	Hyperglycaemia	1
Falls	4	Hypotension	1
Cognitive function decline	3	Inguinal hernia	1
Gastroenteritis	3	Ligament rupture	1
Gastro-oesophageal reflux	3	Mobility decreased	1
Lower respiratory tract infection	3	Neuropathy (ulnar)	1
Urinary obstruction	3	Obstructive sleep apnoea	1
Acute kidney injury	2	Odynophagia	1
Anaemia (iron deficiency)	2	Pain (hip)	1
Aspiration pneumonia	2	Pain (lower back)	1
Asthma exacerbation	2	Pain (shoulder)	1
Bleeding (gastrointestinal)	2	Postural hypotension	1
Cancer (lungs, metastatic)	2	Presyncope	1
Cholelithiasis	2	Schizophrenia	1
Delirium	2	Shortness of breath	1
Faecal abnormalities	2	Spinal cord compression	1
Gastritis	2	Transurethral resection of prostate	1
Hypoglycaemia	2	Trauma (musculoskeletal)	1
Neuropathy (peripheral)	2	Tremors	1
Pain (knee)	2	Ulcerative oesophagitis	1
Seizures	2	Ulcers (duodenal)	1
Sepsis	2	Venous thromboembolism	1
Syncope	2	Vertigo	1
Vasectomy	2		
Bleeding (postmenopausal)	1		
Cancer (brain tumour)	1		
Cancer (breast, metastatic)	1		
Celiac disease	1		
Cholecystitis	1		
Colitis	1		
Diabetic ketoacidosis	1		
Diarrhoea and vomiting	1		
Discitis	1		

Appendix 13: Customised data collection form for research work in Chapter Five of thesis

Hospital data collection - Investigating the relationship between of statins and skin infections.

Charlson index weight	Age:	Patient number: Case / Control (matched to _____)
0 1 2 3 4 5	☐ 0-49 years ☐ 50-59 years ☐ 60-69 years ☐ 70-79 years ☐ 80-89 years ☐ 90-99 years	**Index date:** **Diagnosis:** _____ **(ICD-10 code)** ☐ L00-L08 Infections of the skin and subcutaneous tissue ☐ L10-L14 Bullous disorders ☐ L20-L30 Dermatitis and eczema ☐ L40-L45 Papulosquamous disorders ☐ L50-L54 Urticaria and erythema ☐ L55-L59 Radiation-related disorders of the skin and subcutaneous tissue ☐ L60-L75 Disorders of skin appendages ☐ L80-L99 Other disorders of the skin and subcutaneous tissue
	Clinical condition(s) / Risk factors	**Medication** **Dose & Freq**
1	☐ Cerebrovascular disease ☐ *Chronic liver diseases or cirrhosis* ☐ Chronic pulmonary disease ☐ Congestive cardiac insufficiency ☐ Connective tissue disease ☐ Dementia ☐ *Diabetes (slight, without complications)* ☐ Myocardial infarct ☐ Peripheral vascular disease ☐ Ulcers	☐ Atorvastatin ☐ Fluvastatin ☐ Pravastatin ☐ Rosuvastatin ☐ Simvastatin
2	☐ ***Diabetes*** *with complications* ☐ Hemiplegia ☐ Leukemia ☐ Lymphoma ☐ Renal disease (moderate/severe) ☐ *Tumors*	☐ Antiplatelets: ☐ ACEI/ARB: ☐ Beta blockers:
3	☐ *Liver disease (moderate/severe)*	☐ amoxicillin+clavulanic acid ☐ phenoxymethylpenicillin ☐ clindamycin ☐ cephalexin ☐ cefuroxime ☐ doxycycline ☐ minocycline ☐ azithromycin ☐ clarithromycin ☐ erythromycin ☐ roxithromycin ☐ ciprofloxacin ☐ moxifloxacin
6	☐ *Tumors (malignant/metastasis)* ☐ AIDS --- ☐ **Obesity (BMI = _____)** ☐ **Smoking** OTHERS:	☐ Immunosuppressants: ☐ Corticosteroids: OTHERS:
--- **Charlson Index Score**	**Microorganism(s)/infection site:**	

Appendix 14: Ethics approval from the South Metropolitan Area Health Service for research work in Chapter Five of thesis

Government of **Western Australia**
South Metropolitan Health Service

16 December 2016

Mr Humphrey Ko
Pharmacy
Rockingham General Hospital
B Block Ground Floor
Elanora Drive
ROCKINGHAM WA 6168

Dear Humphrey

Project Title:	***Investigating the Relationship Between Statins and Skin Infections***
REG Number:	**2012-285**
HREC:	**South Metropolitan Health Service Human Research Ethics Committee (EC00265)**
SMHS Site:	**Rockingham Peel Group**

The following **amendment** has been **approved** by the South Metropolitan Health Service (SMHS) Human Research Ethics Committee and participating SMHS sites:

Amendment
Extension of approval date from 18 April 2017 to 30 June 2018.

Please submit a copy of this approval letter to the Research Governance office at other participating sites that are under this HREC approval (if any).

Yours sincerely

Wendy Khoo
Delegate of the Chair
South Metropolitan Health Service HREC
A/Research Governance Officer

Research Ethics and Governance
South Metropolitan Health Service
Locked Bag 100, PALMYRA DC WA 6961
Telephone: (08) 6151 1180
Email: SMHS.REG@health.wa.gov.au
www.southmetropolitan.health.wa.gov.au

Appendix 15: Reciprocal ethics approval for research from Curtin University

MEMORANDUM

Curtin University

To:	Prof Jeffery Hughes School of Pharmacy
CC:	
From	Professor Peter O'Leary, Chair HREC
Subject	Reciprocal ethics approval Approval number: HR155/2015
Date	10-Aug-15

Office of Research and Development
Human Research Ethics Office

TELEPHONE 9266 2784
FACSIMILE 9266 3793
EMAIL hrec@curtin.edu.au

Thank you for your application submitted to the Human Research Ethics Office for the project: 6188
Investigating the relationship between statins and skin infections

Your application has been approved through Curtin University Human Research Ethics Committee (HREC) through a reciprocal approval process with the lead HREC.

The lead HREC for this project has been identified as South Metopolitan Area Health Service HREC

Approval number from the lead HREC is noted as: 12/285

Please note the following conditions of approval:

1. Approval is granted from **11-Aug-15** to **18-Oct-15**
2. Research must be conducted as stated in the approved protocol.
3. Any amendments to the approved protocol must be approved by the Ethics Office.
4. An annual progress report must be submitted to the Ethics Office annually, on the anniversary of approval.
5. All adverse events must be reported to the Ethics Office.
6. A completion report must be submitted to the Ethics Office on completion of the project.
7. Data must be stored in accordance with WAUSDA and Curtin University policy.
8. The Ethics Office may conduct a randomly identified audit of a proportion of research projects approved by the HREC.

Should you have any queries about the consideration of your project please contact the Ethics Support Officer for your faculty, or the Ethics Office at hrec@curtin.edu.au or on 9266 2784. All human research ethics forms and guidelines are available on the ethics website.

Yours sincerely,

Professor Peter O'Leary
Chair, Human Research Ethics Committee

Appendix 16: Supplementary hospital data showing the relationship between statin use with obesity and diabetes respectively

Appendix 16-1: Supplementary hospital data showing the relationship between statin use with obesity[‡]

	Cases only subgroup (with SSTIs), *n = 165*			Controls only subgroup (without SSTIs), *n = 165*		
Variable	**Obese (%) *n = 52***	**Non-obese (%) *n = 113***	**Relative risk[#] (95% CI) and p-value**	**Obese (%) *n = 24***	**Non-obese (%) *n = 141***	**Relative risk[#] (95% CI) and p-value**
Drug exposure on admission						
Atorvastatin						
Users	7 (13.5)	21 (18.6)	0.761	1 (4.2)	27 (19.1)	0.213
Non-users	45 (86.5)	92 (81.4)	(0.384 – 1.510) p = 0.507	23 (95.8)	114 (80.9)	(0.030 – 1.511) p = 0.082
Pravastatin						
Users	2 (3.8)	4 (3.5)	1.060	0 (0)	4 (2.8)	Nil pravastatin users in obese group
Non-users	50 (96.2)	109 (96.5)	(0.334 – 3.363) p = 1.000	24 (100)	137 (97.2)	
Rosuvastatin						
Users	2 (3.8)	8 (7.1)	0.620	3 (12.5)	14 (9.9)	1.244
Non-users	50 (96.2)	105 (92.9)	(0.176 – 2.187) p = 0.507	21 (87.5)	127 (90.1)	(0.414 – 3.739) p = 0.717
Simvastatin						
Users	3 (5.8)	7 (6.2)	0.949	2 (8.3)	6 (4.3)	1.784
Non-users	49 (94.2)	106 (93.8)	(0.358 – 2.515) p = 1.000	22 (91.7)	135 (95.7)	(0.505 – 6.297) p = 0.329

([‡]) Due to the small sample size of each subgroup, variables were stratified into a 2 x 2 contingency table and two-sided Fisher's exact test was conducted. ([#]) Relative risk was calculated due to samples being taken from an independent sample.

Appendix 16-2: Supplementary hospital data showing the relationship between statin use with diabetes[‡]

	Cases only subgroup (with SSTIs), *n = 165*			Controls only subgroup (without SSTIs), *n = 165*		
Variable	**Diabetics (%) *n = 38***	**Non-diabetics (%) *n = 127***	**Relative risk[#] (95% CI) and p-value**	**Diabetics (%) *n = 39***	**Non-diabetics (%) *n = 126***	**Relative risk[#] (95% CI) and p-value**
Drug exposure on admission						
Atorvastatin						
Users	14 (36.8)	14 (11)	2.854	9 (23.1)	19 (15.1)	1.468
Non-users	24 (63.2)	113 (89)	(1.699 – 4.795) p = 0.001	30 (76.9)	107 (84.9)	(0.786 – 2.740) p = 0.328
Pravastatin						
Users	1 (2.6)	5 (3.9)	0.716	1 (2.6)	3 (2.4)	1.059
Non-users	37 (97.4)	122 (96.1)	(0.117 – 4.382) p = 1.000	38 (97.4)	123 (97.6)	(0.190 – 5.915) p = 1.000
Rosuvastatin						
Users	2 (5.3)	8 (6.3)	0.861	10 (25.6)	7 (5.6)	3.002
Non-users	36 (94.7)	119 (93.7)	(0.241 – 3.073) p = 1.000	29 (74.4)	119 (94.4)	(1.795 – 5.022) p = 0.001
Simvastatin						
Users	3 (7.9)	7 (5.5)	1.329	4 (10.3)	4 (3.2)	2.243
Non-users	35 (92.1)	120 (94.5)	(0.493 – 3.578) p = 0.698	35 (89.7)	122 (96.8)	(1.057 – 4.758) p = 0.091

([‡]) Due to the small sample size of each subgroup, variables were stratified into a 2 x 2 contingency table and two-sided Fisher's exact test was conducted. ([#]) Relative risk was calculated due to samples being taken from an independent sample.