Clarifying the role of pharmacological factors in the pathogenesis of statin-associated muscle symptoms and the ability of geranylgeranyl pyrophosphate to prevent statin-induced muscle fatigue

by

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Thesis
Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

School of Health, Medical and Applied Sciences
Central Queensland University

November 2019

Candidate's Statement: By submitting this thesis for formal examination at CQUniversity Australia, I declare that it meets all requirements as outlined in the Research Higher Degree Theses Policy and Procedure.

Abstract

Background

The onset of statin-associated muscle symptoms (SAMS) can reduce quality of life in affected individuals, and also presents a significant obstacle for maintaining compliance with these pharmaceuticals. More effective strategies for managing the various clinical manifestations of SAMS are required; however, identifying such therapies has been complicated by the multifaceted and variable aetiology of statin-induced myotoxicity. Much ambiguity remains surrounding the molecular mechanisms underlying the different forms of SAMS, as well as the role of pharmacological factors in the pathogenesis of these conditions. It has been suggested that the co-administration of geranylgeranyl pyrophosphate (GGPP) with statins may prevent myotoxic effects; however, this is yet to be thoroughly investigated *in vivo*. Accordingly, the aim of this thesis was to clarify the role of statin lipophilicity and dose in the pathogenesis of SAMS, as well as to determine the feasibility of GGPP repletion to prevent statin-induced myotoxicity *in vivo*.

Methods

In order to meet these objectives, a systematic-review and meta-analysis was conducted, as well as a series of rodent-based studies. Data for the meta-analysis was obtained from 135 randomised controlled trials which reported information on SAMS. The animal studies included a series of functional, molecular and biochemical analyses aimed at assessing the impact of statin and/or GGPP administration (in the form of geranylgeraniol) on skeletal muscle integrity. Additionally, cardiac and vascular smooth muscle performance was also assessed to: (i) determine if these parameters were significantly altered by the presence of

SAMS and; (ii) establish whether GGPP repletion was associated with adverse changes in these tissues.

Results

The systematic review and meta-analysis found that neither statin lipophilicity nor dose had a significant influence on the frequency of SAMS in randomised controlled trials. Conversely, the results of the rodent-based studies demonstrated that treatment with a high-dose lipophilic statin was associated with greater myotoxic effects compared to low-dose or hydrophilic formulations. This work also identified that the molecular mechanisms which underlie milder forms of SAMS differ significantly from the more severe manifestations. Additionally, it was observed that GGPP administration (in the form of geranylgeraniol) was able to prevent the myotoxic effects of statins without causing adverse changes in cardiovascular performance.

Conclusion

The findings of this study suggest that both lipophilicity and dose can influence the myotoxic potential of statins; however, the significance of this effect may be altered in the presence of other risk factors for SAMS. This work also demonstrates that GGPP repletion is a feasible intervention for preventing statin-induced skeletal muscle damage *in vivo*. In turn, the findings of this project have significant implications for elucidating the pathogenesis, and potential treatment, of SAMS.

Personal Acknowledgements

There is a constellation of individuals whose time, assistance and support has facilitated the progression of this thesis from “distant aspiration” to “frank reality”. Accordingly, I would like to gratefully acknowledge the following groups/individuals: my Family (Rod, Zoe, Shea, Sidney and Mab); Supervisors (Rebecca and Andrew); Lab mates (Gina, Kim and Kylie) and CQU Lab technicians (Judy, Giselle, Nicole, Ingrid, Paul, Charmain, Heather and Tania).

All I can say is thank you.

Declaration of Authorship and Originality

By submitting this thesis for formal examination at CQUniversity Australia, I declare that all of the research and discussion presented in this thesis is original work performed by the author. No content of this thesis has been submitted or considered either in whole or in part, at any tertiary institute or university for a degree or any other category of award. I also declare that any material presented in this thesis performed by another person or institute has been referenced and listed in the reference section.

Additional Note: Owing to the presence of published material in this thesis, as well as several reference lists, the Turnitin similarity score for this document is quite high. Nonetheless, all of the work presented in this thesis is the original work of the candidate. Furthermore, any material obtained from other sources has been referenced appropriately.

Copyright Statement

Acknowledgement of Financial Support

This RHD candidature was supported under the Commonwealth Government's Research Training Scheme. I gratefully acknowledge the financial support provided by the Australian Government. I also gratefully acknowledge the funding received from the Australian Government through the Research Training Scheme Stipend. This research was also undertaken with in-kind support of materials and equipment provided by the Central Queensland University Pharmacology Research Laboratory.

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Publications and Presentations by the Candidate Relevant to the Thesis

Manuscripts submitted and published

Irwin JC, Khalesi S, Fenning AS, Vella RK. The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis. Pharmacol Res. 2018;128:264-273. (Chapter 2)

Irwin JC, Fenning AS, Ryan KR & Vella RK. Validation of a clinically-relevant rodent model of statin-associated muscle symptoms for use in pharmacological studies. Toxicol Appl Pharmacol. 2018;360;78-87. (Chapter 3)

Irwin JC, Fenning AS, Vella RK. Geranylgeraniol prevents statin-induced skeletal muscle fatigue without causing adverse effects in cardiac or vascular smooth muscle performance. Transl Res. Forthcoming 2019. (Chapter 6)

Manuscripts submitted and under review

Irwin JC, Fenning AS, Vella RK. Gene expression profiles in statin-treated rodents with and without myalgia. (Chapter 4)

Irwin JC, Fenning AS & Vella RK. Statins with different lipophilic indices exert distinct effects in skeletal, cardiac and vascular smooth muscle. Manuscript submitted for publication. (Chapter 5)

Conference Presentations

Irwin JC. The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis of randomised controlled trials. Conference presented at 65th Annual Scientific Meeting of the Cardiac Society of Australia and New Zealand; 2017; Perth.

Administration of geranylgeraniol in a rodent model of statin-induced myalgia prevents skeletal muscle damage without adversely affecting cardiovascular performance. Conference presented at 67th Annual Scientific Meeting of the Cardiac Society of Australia and New Zealand; 2019; Adelaide.

List of Abbreviations

ANOVA	Analysis of variance
APA	Action potential amplitude
APD	Action potential duration
ATP	Adenosine triphosphate
BMI	Body mass index
BNP	Brain natriuretic peptide
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CI	Confidence intervals
CK	Creatine kinase
CK-MM	Creatine kinase MM fraction
CRC	Concentration-response curve
Ctsl	Cathepsin L
CO_2	Carbon dioxide
CON	Control
CoQ10	Coenzyme Q10
CVD	Cardiovascular disease
EC50	Concentration required to obtain half-maximal response
EF50	Frequency required to obtain half-maximal response
eNOS	Endothelial nitric oxide synthase
FC	Force of contraction

FFC	Force-frequency curve
FFP	Farnesyl pyrophosphate
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gastrocnemius
GGOH	Geranylgeraniol
GGPP	Geranylgeranyl pyrophosphate
GTPases	Small guanosine triphosphate-binding proteins
HDL	High-density lipoprotein
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
H-FABP	Heart-type fatty acid binding protein
KCl	Potassium chloride
KHB	Krebs-Henseleit buffer
Kim1	Kidney injury molecule-1
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
Max +dP/dt	Maximum rate of contraction
Max -dP/dt	Maximum rate of relaxation
MG	Myoglobin
MHC	Myosin heavy chain
MHC-β	Myosin heavy chain beta
mRNA	Messenger ribonucleic acid
MuRF-1	Muscle RING-finger protein-1
Mstn	Myostatin
Mt1a	Metallothionein 1A

MTD	Maximum tolerable dose
Nox2	Nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase 2
O_2	Oxygen
Pdk4	Pyruvate dehydrogenase kinase 4
Pgc-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pparα	Peroxisome proliferator-activated receptor α
PRAV	Pravastatin
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSPERO	International Prospective Register for Systematic Reviews
RCT	Randomised controlled trial
RMP	Resting membrane potential
ROS	Reactive oxygen species
ROUT	Robust regression and outlier removal
RR	Risk ratio
RT-qPCR	Real-time quantitative polymerase action / Quantitative reverse transcription polymerase chain reaction
SAMS	Statin-associated muscle symptoms
SD	Standard deviation
SIM	Simvastatin
Sod1	Superoxide dismutase 1
Sod2	Superoxide dismutase 2
SOL	Soleus
TA	Tibialis anterior
TPSS	Tyrode's physiological salt solution
TR90	Time to 90% relaxation

Ucp3	Uncoupling Protein 3
ULN	Upper limit of normal
VLDL	Very low-density lipoprotein

CHAPTER 1

Introduction and Statement of Research Aim/Objectives

Preamble

This chapter details the background information related to this project, as well as the current gaps in knowledge which this work aimed to address. Following this discussion, the aims and objectives of this project are listed. This chapter concludes with a brief overview of the presentation of this thesis.

The Vancouver style of referencing has been used in this chapter owing to its preference as a referencing style in the Medical Sciences.

1. Introduction

1.1. Cholesterol and its physiological significance

The biomolecule, cholesterol, serves as the major lipid component of cell membranes, as well as the precursor of steroid hormones and bile [1]. Due to its physiological importance, almost all of the body's cells require a continuous supply of cholesterol [2]. This requirement is met through an array of biosynthetic, transportation and regulatory mechanisms [3]. Cholesterol is primarily obtained via endogenous biosynthesis in the hepatocytes of the liver, though it can also be acquired from dietary or biliary sources [4]. Once in circulation, this lipid is transported around the body using a series of lipoproteins (including high-density lipoprotein (HDL) and very low-density lipoprotein (VLDL)) [5]. Principally, cholesterol is carried by low-density lipoprotein (LDL) which moves it from the liver to the peripheral tissues [6].

1.2. Hypercholesterolemia

Although adequate cholesterol levels are essential for normal physiology, hypercholesterolemia (i.e. average plasma cholesterol $\geq$ 5 mmol/L and/or LDL-cholesterol $\geq$ 3 mmol/L) is a significant risk factor for atherosclerotic cardiovascular disease, particularly coronary heart disease (CHD) and stroke [7]. Effective management of hypercholesterolemia is thus imperative for preventing adverse cardiovascular events [8, 9]. Cholesterol-lowering relies on a combination of dietary management, exercise plans and pharmaceuticals [10-12]. With regards to the latter, the first-line pharmacological therapy is 3-hydroxy-3-

methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, otherwise known as statins [13].

1.3. Statins and their physiological effects

Statins suppress endogenous cholesterol synthesis by inhibiting the enzyme, HMG-CoA reductase, which catalyses the rate-limiting step in mevalonate pathway (**Fig. 1**) [13]. The reduction in intracellular cholesterol concentration subsequently stimulates an increase in the expression of LDL receptors in both hepatic and peripheral tissues [14]. This alteration results in enhanced removal of LDL-cholesterol from the bloodstream, and thus, a reduction in circulating cholesterol levels [15].

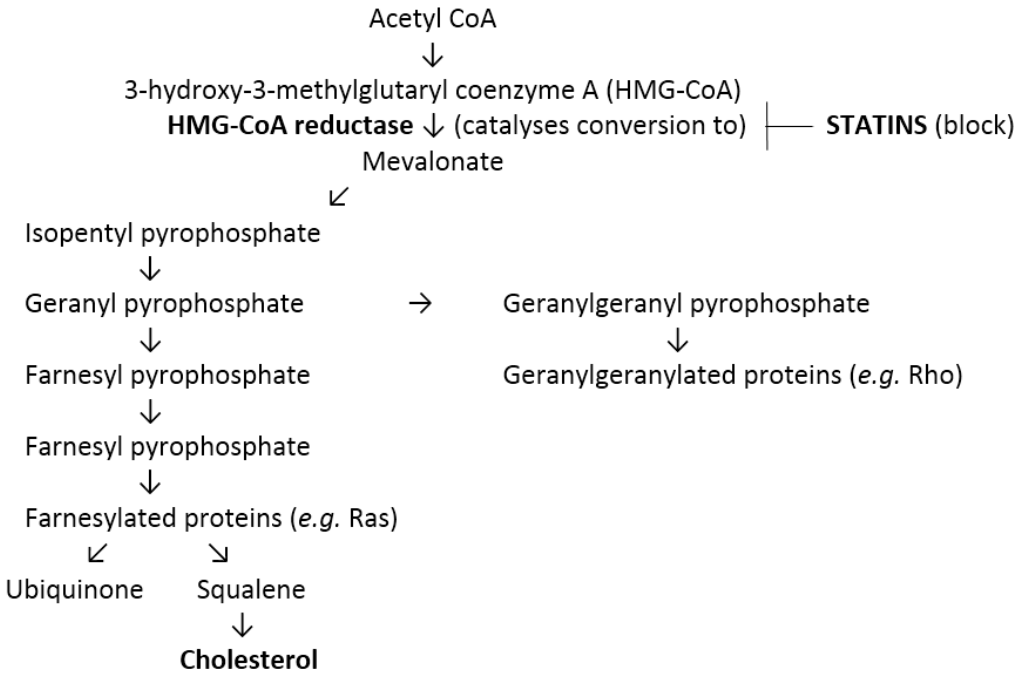

Fig. 1 The mevalonate pathway is responsible for endogenous cholesterol synthesis (adapted from Farnier and Davignon [12]).

There are currently seven statins available for clinical use: atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin [16]. As a class, statins are associated with a 20-30% reduction in both total and LDL-cholesterol levels [17]. In turn, these medications are estimated to reduce the risk of major vascular events by 21% for every 1.0 mmol/L reduction in serum LDL-cholesterol concentration [18]. Consequently, statins have become a mainstay intervention for the prevention of CHD and stroke [13].

The cardiovascular benefits conferred by statins are derived not only from their cholesterol-lowering capacity, but also their pleiotropic effects [19]. Such responses include abrogation of endothelial dysfunction, anti-oxidative effects, modulation of thrombosis as well as inhibition of inflammatory activity [20, 21]. Nonetheless, the cardioprotective effects of these medications can only be fully realised when compliance is adequately maintained [22]. The rate of statin discontinuation, however, is considerable with an estimated 40-75% of individuals ceasing therapy within 24 months of initiating treatment [23, 24]. Noncompliance with statins is associated with an increased risk of adverse cardiovascular events [23, 25, 26], and thus presents a considerable obstacle for improving health outcomes. Several factors are postulated to prompt statin discontinuation, the most significant of these being the development of adverse side effects [27].

1.4. Statin-associated side effects

Statins are generally well-tolerated, yet in some individuals they are associated with negative side effects such as musculoskeletal problems, hepatotoxicity, new-onset type II diabetes mellitus and/or impaired cognition [28, 29]. Estimates of the frequency of these side effects

vary [30], but it is consistently reported that the most common are adverse statin-associated muscle symptoms (SAMS) [31, 32]. Nevertheless, the data currently available on the frequency and development SAMS is conflicting, and this has led to controversy surrounding the prevalence of these conditions [33, 34].

The term "SAMS" encompasses several distinct muscle-related pathologies which can be induced following treatment with statins [35]. Each form of SAMS differs in terms of severity, the presence/absence of elevated creatine kinase (CK) levels (a serum biomarker of skeletal muscle damage) and whether muscle pain/fatigue is experienced [36]. Various terms are used to describe the individual types of SAMS including: asymptomatic CK elevation, myalgia, myopathy, myositis, rhabdomyolysis/severe myonecrosis and autoimmune-mediated necrotising myositis/myopathy [29, 37]. Problematically, there are currently no commonly accepted definitions for these terms [11, 38], and they are often used interchangeably [39]. Several panels have tried to standardise these definitions [32, 38, 40-42]; however, there is still no universally recognised set of classifications. For the purpose of this work, the following terminology (adapted from Selva-O'Callaghan et al. [39]) has been used for describing the variants of SAMS:

- Asymptomatic CK elevations – serum CK levels $\geq$ 10 × upper limit of normal in the absence of muscle pain/weakness/fatigue.
- Myalgia/mild myopathy – muscle pain/weakness/fatigue in the absence of significant elevations in serum CK (e.g. nil change or increase $\leq$ 5 × upper limit of normal).
- Myositis or immune-mediated necrotising myopathy – muscle pain/weakness/fatigue accompanied by elevated serum CK (e.g. $\geq$ 10 × upper limit of normal) and evidence of necrosis with immune-mediated features (i.e. infiltration of inflammatory cells).

- Rhabdomyolysis/severe myonecrosis – exceptionally high serum CK elevations (e.g. $\geq$ 100 × upper limit of normal) accompanied by myoglobinuria and renal impairment.

In the absence of standardised definitions, the accuracy with which particular types of SAMS can be diagnosed in the clinical setting is impeded [43]. This point is further exacerbated by the fact that there are currently no "gold standard" criteria for detecting or monitoring SAMS [43]. Ultimately, uncertainty in the identification and diagnosis of SAMS has summated in ambiguity concerning the prevalence of these conditions [44, 45]. This fact is principally exemplified by the disparity in reports from randomised controlled trials (RCTs) and clinical practice. For instance, the frequency of SAMS in RCTs ranges from 1.5-3% [46], while rates of 10-25% are commonly reported in observational cohort studies [28, 47] (further discussion is provided in Chapter 2). In turn, uncertainty about the frequency of SAMS has prompted debate about the role of the nocebo effect (i.e. adverse events resulting from expressed/internal expectations of harm) on the rate of SAMS reported in the clinical setting [24, 48]. Regardless of its exact frequency, the onset of SAMS can significantly impact upon the quality of life of affected individuals by causing pain and/or difficulty completing daily tasks (e.g. opening jars) [49, 50]. Furthermore, by prompting statin discontinuation, SAMS presents a significant obstacle for improving cardiovascular outcomes [24, 39, 45, 51].

1.5. Current knowledge on the pathogenesis of SAMS

Presently, the management of SAMS is limited to altering statin treatment to less rigorous therapy (e.g. lowering statin dose / switching to alternative-day dosing), or ceasing statin use entirely if symptoms are intolerable [31]. While these measures are effective in alleviating SAMS, they can also compromise the cardioprotective effects of statins [52, 53].

Consequently, alternative therapies which can successively manage SAMS, without impacting upon the cardioprotective effects of these medications, need to be identified. In order to achieve this outcome, a thorough understanding of the mechanisms underlying statin-induced myotoxicity is required. Although this subject has been widely studied [54], there remains significant ambiguity concerning the pathogenesis of SAMS [55, 56].

The molecular alterations which occur during statin-induced myotoxicity are complex and appear to be influenced by genetic and immunological factors [13, 57]. Several hypotheses concerning the mechanisms underlying SAMS are reported in the literature. Such factors include mitochondrial dysfunction [58, 59], induction of atrophy-related genes (e.g. *Atrogin-1*) [60], increased oxidative stress [61], alterations in intracellular calcium homeostasis [62] as well as impaired metabolism [63] (**Fig. 2**). Although these effects are commonly observed during SAMS, not all data supports their involvement. For example, while statin treatment has been shown to reduce mitochondrial volume [64, 65] and disrupt respiration [66], there are also cases of SAMS occurring in the absence of significant mitochondrial dysfunction [67].

This variability in reports may relate to the fact that mechanistic studies of statin-induced myotoxicity are generally not distinguished according to the specific form of SAMS being investigated. There is increasing evidence that the various types of SAMS do not present as a continuum of symptoms, but rather are distinct conditions (albeit related) with their own aetiologies [29, 42]. Mechanistic studies of SAMS, however, have typically neglected this point and their findings are often generalised to all forms of statin-induced myotoxicity. This stereotyping may be preventing the identification of individual factors which are specific for the development of particular types of SAMS.

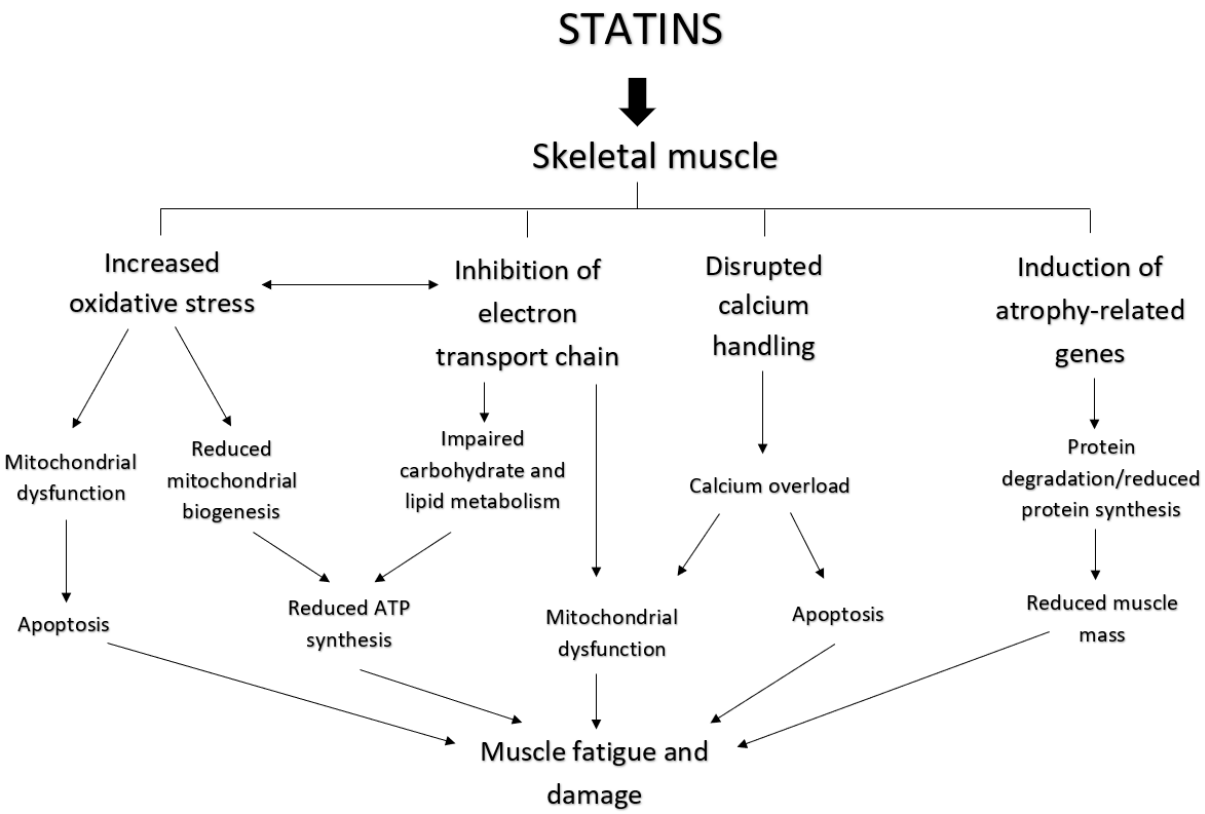

Fig 2. Schematic summarising the cellular changes typically associated with SAMS.

1.6. Role of pharmacological factors in SAMS

As alluded to above, there is growing recognition that the pathogenesis of statin-induced myotoxicity is highly individualised [68]. Several patient-related risk factors have been associated with an increased likelihood of developing SAMS, including female sex, old age (i.e. > 75 years) and genetic mutations in hepatic transport proteins [24, 69, 70]. Again, however, data pertaining to the role of each of these factors in the aetiology of SAMS is inconsistent (further discussion is provided in Chapter 2). In addition to patient-orientated factors, the pharmacological characteristics of statins may also play a key role in their myotoxic potential [29, 71]. While all statins are inhibitors of HMG-CoA reductase, they each vary in their metabolism, bioavailability and efficacy [14, 72, 73]. Additionally, lipid

solubility (i.e. lipophilicity) also differs significantly between the different formulations of these medications. Specifically, pravastatin and rosuvastatin are classed as hydrophilic statins, while the other preparations are designated as lipophilic [74]. Hydrophilic statins are generally referred to as being "hepatoselective" as they rely on specific transporters to be transported into hepatocytes [72]. Conversely, lipophilic statins are postulated to be capable of diffusing non-selectively into extra-hepatic tissues, such as skeletal muscle [71, 75] (further discussion is provided in Chapter 5). Accordingly, there is evidence to suggest that lipophilic statins are associated with a greater risk of SAMS [29, 35, 54]. The frequency of SAMS is also suggested to be greater in persons taking high-dose statins (i.e. $\geq$ 40 mg), owing to the potential for higher (and thus more toxic) plasma concentrations of these drugs to be reached [57, 76]. This evidence has encouraged the practice of switching statin prescriptions from high-dose to low-dose therapy, or from a lipophilic statin to a hydrophilic statin, in persons with SAMS [24, 27]. Nonetheless, not all data supports that these factors have a significant effect on the likelihood of statin-induced myotoxicity [37, 77, 78] (further discussion is provided in Chapter 2). Clarifying the influence of lipophilicity and dose on the pathogenesis of SAMS is thus essential for elucidating the mechanisms underlying these conditions.

1.7. Role of geranylgeranyl pyrophosphate in SAMS

While debate remains concerning the molecular mechanism underlying SAMS, it is generally agreed that the myotoxic effects of statins is related, at least in part, to their inhibition of the mevalonate pathway [71, 79, 80]. In addition to manufacturing cholesterol, the mevalonate pathway synthesises several other compounds including coenzyme Q10 (CoQ10), dolichols, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) [44, 81]. It was

originally suggested that depletion of CoQ10 was central to the development of SAMS (this was owing to its role in the mitochondrial respiratory chain and the prevalence of mitochondrial dysfunction during statin-induced myotoxicity) [82]. The inability of CoQ10 supplementation to consistently alleviate SAMS in clinical trials, however, has cast doubt over its importance in the development of these conditions [83, 84]. Alternatively, increasing evidence from cell culture studies implicates the depletion of GGPP as a major contributor to statin-induced myotoxicity [85-89] (**Fig. 3**; further discussion is provided in Chapter 6). This point is exemplified by the fact that administration of this compound to statin-treated skeletal myocytes has been shown to reverse mitochondrial dysfunction and *Atrogin-1* expression [90, 91].

The ability of GGPP repletion to prevent statin-induced muscle damage *in vivo* is yet to be thoroughly investigated. Further investigation into the effects of GGPP *in vivo* is required, not only to verify the results from the cell culture studies, but also to ensure that supplementation with this compound will not have adverse effects on the cardioprotective properties of statins. Indeed, some pleiotropic effects of statins have been linked to reduced activity of small GTP-binding proteins (i.e. small GTPases) as a consequence of GGPP depletion in cardiovascular tissues [92, 93] (**Fig. 3**; further discussion is provided in Chapter 6). Hence, it is important to establish whether GGPP repletion can alleviate the myotoxic effects of statins *in vivo* without limiting the cardioprotective effects conferred by these medications.

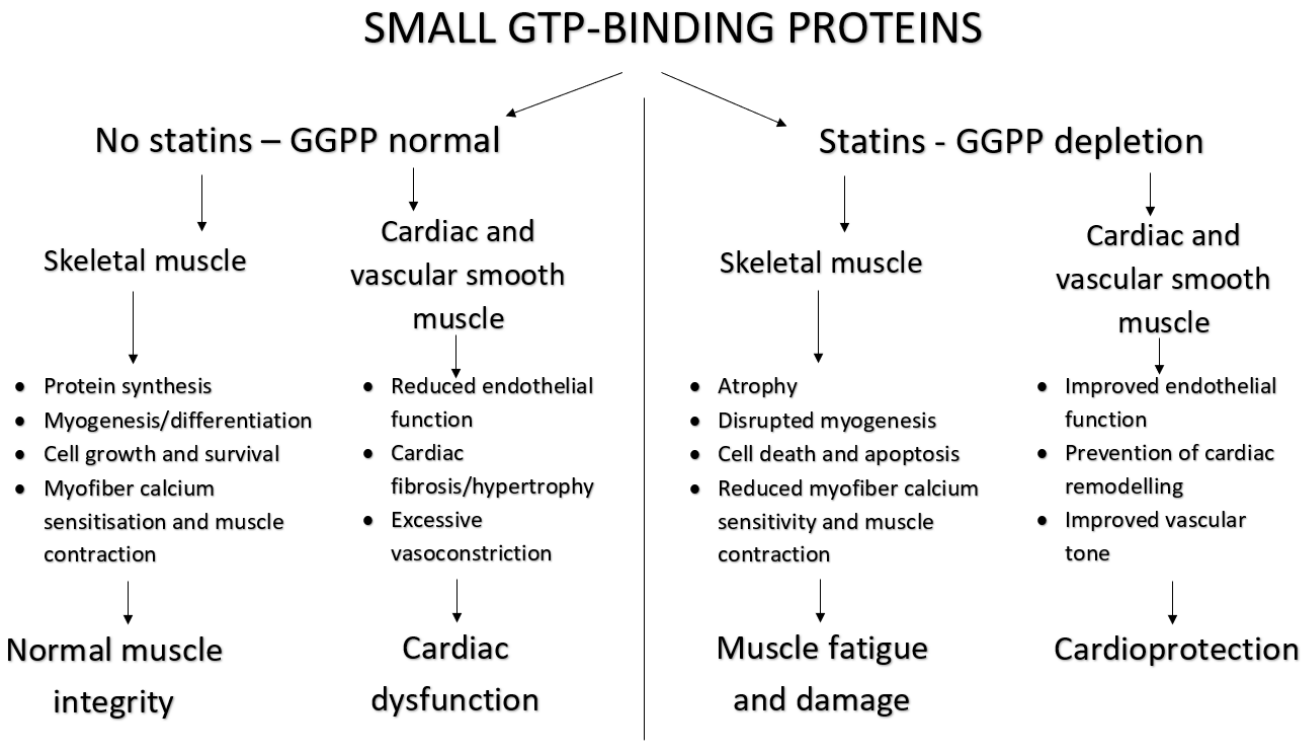

Fig 3. Comparison of normal and inhibited effects of geranylgeranylated small GTP-binding proteins in skeletal muscle and cardiovascular tissues.

1.8. Cardiovascular performance during SAMS

As noted above, the molecular mechanisms which underlie the various forms of SAMS remain to be fully elucidated. However, even less is known about the changes which occur to the myocardium and vasculature in the presence of statin-induced skeletal muscle damage. Statins are reported to exert a differential effect on cardiac and skeletal muscle, particularly in terms of free radical production and mitochondrial biogenesis [58]. Nonetheless, the potential for the effects of statins on myocardial and vascular performance to be altered during SAMS has not been rigorously assessed.

The release of intramuscular constituents following skeletal muscle damage (particularly rhabdomyolysis) has the potential to induce fatal arrhythmias [94, 95]. Moreover, there is evidence that, under certain conditions, statins can exert a direct toxic effect on cardiac and vascular smooth muscle [96, 97]. Although it is assumed that the cardioprotective effects of statins are maintained during SAMS (particularly in the milder variants of these conditions) [31], quantitative evidence to support this presumption would improve evidence-based practice in the management of SAMS.

2. Project aim and objectives

Considering the knowledge gaps identified above, this project aimed to:

Clarify the role of statin lipophilicity and dose in the pathogenesis of SAMS, and to evaluate the ability of GGPP repletion to prevent statin-induced myotoxicity *in vivo*.

In order to meet this aim, the following research objectives were addressed:

i. Clarify the influence of statin dose and lipophilicity on the pathogenesis of SAMS.
ii. Assess the ability of GGPP administration (in the form of geranylgeraniol) to prevent SAMS *in vivo*.
iii. Evaluate cardiac and vascular smooth muscle performance in the presence of SAMS.

3. Presentation of thesis

This thesis is presented as a combination of one systematic review/meta-analysis and four original research papers (the publication status of which is indicated at the start of each respective chapter). Together, these papers describe the methods, results and discussions of the five studies conducted as part of this project. The following points provide an outline of the chapters contained within this work. The beginning of each chapter also contains a "Preamble" which describes how the studies presented in this thesis build upon one another to form a coherent body of work.

Thesis outline:

- Chapter 2 reports the results of a systematic review and meta-analysis investigating the effect of lipophilicity and dose on the frequency of SAMS reported in RCTs (research objective i).
- Chapter 3 presents a validation study aiming to identify a reproducible and clinically-relevant rodent model of SAMS for use in mechanistic studies (this model was subsequently used in the investigations presented in Chapters 4-6).
- Chapter 4 details the findings of a dose-response study which assessed the functional, biochemical and molecular changes which occur in statin-treated rats with and without myalgia (research objective i).
- Chapter 5 reports the results of a comparative study aiming to establish whether hydrophilic and lipophilic statins exert different or comparable effects on skeletal, cardiac and vascular smooth muscle performance (research objectives i and iii).
- Chapter 6 describes a study which assessed the feasibility of GGPP administration (in the form of geranylgeraniol) to prevent statin-induced myalgia *in vivo* (research objectives ii and iii).

- Chapter 7 is an extended discussion which reiterates the significance and originality of the work presented in this thesis. This chapter also discusses the project's limitations and provides directions for future research which will build upon the knowledge generated from this work.
- Chapter 8 presents a summary / conclusion of the research undertaken as part of this project.

Reference lists for individual manuscripts/sections in this thesis are provided at the end of each respective chapter. Unless otherwise stated, the referencing style used per chapter is formatted in accordance with the corresponding journal specifications.

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CHAPTER 2

The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis

Preamble

A primary objective of this work was to clarify the role of lipophilicity and dose in the pathogenesis of statin-induced myotoxicity. In order to appraise the existing evidence available on this topic, a systematic review and meta-analysis was conducted. Data from randomised controlled trials was used as meta-analyses of these studies remain the "gold-standard" for assessing drug-induced effects. The results of this meta-analysis elucidated key themes concerning the role of statin lipophilicity and dose in SAMS. In turn, this study assisted in contextualising the significance/potential impact of the findings generated from this project.

This chapter contains the manuscript titled, "The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis", which has been published in *Pharmacological Research* (2018;128:264-273). This work also includes Supplementary data which is not presented in the manuscript. The Supplementary files accompanying this paper are provided in Appendix A of this thesis.

Declaration of co-authorship and contribution

Full bibliographic details and Status

Irwin JC, Khalesi S, Fenning AS, Vella RK. The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis. Pharmacol Res. 2018;128:264-273.
Published

Nature of Candidate's Contribution, including percentage of total

JC Irwin (80%): Study conceptualisation and design, acquisition of data, analysis and interpretation of data, writing of original draft, critical revision of manuscript and approval of final version.

Nature of all Co-Authors' Contributions, including percentage of total

S Khalesi (5%): Study concept and design, critical revision of manuscript and approval of final version. AS Fenning (5%): Study concept and design, critical revision of manuscript and approval of final version. RK Vella (10%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Pharmacological Research 128 (2018) 264–273

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Review

The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis

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ARTICLE INFO

Article history:
Received 28 July 2017
Received in revised form
19 September 2017
Accepted 20 September 2017
Available online 21 September 2017

Keywords:
Statin-associated muscle symptoms
Dose
Lipophilicity
Meta-analysis
Randomised controlled trial

ABSTRACT

Addressing the factors which lead to the development of statin-associated muscle symptoms (SAMS) is vital for maintaining patient compliance with these pharmaceuticals, and thus improving patient outcomes. This study aimed to clarify the relationship between statin lipophilicity, or dose, and the frequency of adverse muscle symptoms using a systematic review of randomised controlled trials (RCTs). RCTs, including statin monotherapy and placebo groups, which reported data on muscle adverse events were identified through the PubMed and Scopus databases. Risk ratios (RRs) and 95% confidence intervals (CI) were pooled using a random-effects meta-analysis. A total of 135 RCTs were included in this review. Statin therapy was associated with a significant, but modest, increase in the risk of adverse muscle symptoms compared to placebo (RR = 1.050; 95% CI = 1.014–1.089; $P = 0.007$; $I^2 = 3.291\%$). This significant association was primarily due to the inclusion of RCTs recruiting participants with a history of statin intolerance. Lipophilic statins had no appreciable impact on the development of SAMS compared to hydrophilic formulations. A univariate meta-regression of dose (standardised to atorvastatin dose equivalents) and the risk of musculoskeletal complaints also showed no significant association. The results obtained from this meta-analysis indicate that there is a slight increase in the risk of SAMS, especially in individuals with a history of statin intolerance. There is limited evidence to suggest that the risk of SAMS would differ between the use of lipophilic and hydrophilic statins, or high- and low-dose therapy.

Contents

Abbreviations: BMI, body mass index; CI, confidence intervals; CK, creatine kinase; LDL-C, low-density lipoprotein cholesterol; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; PROSPERO, International Prospective Register for Systematic Reviews; RCTs, randomised controlled trials; RR, risk ratios; SAMS, statin-associated muscle symptoms; ULN, upper limit of normal.

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https://doi.org/10.1016/j.phrs.2017.09.013
1043-6618/

1. Introduction

Statins are the most effective pharmaceuticals for the treatment of hypercholesterolemia and are currently used by an estimated 25 million people worldwide [1–4]. While these medications are safe and well-tolerated, they can cause adverse statin-associated muscle symptoms (SAMS) in some individuals, which in turn, leads to poor patient compliance [5,6]. Statin discontinuation has been shown to cause a near three-fold increase in cardiac event risk, as well as higher rates of all-cause mortality [7,8]. Hence, addressing the factors which lead to the development of SAMS, and maintaining adequate compliance with these pharmaceuticals, is critical for improving patient health outcomes.

SAMS range in severity from mild-to-moderate muscle pain, weakness or fatigue (with or without creatine kinase elevation) to potentially life-threatening rhabdomyolysis [4,9,10]. The exact mechanisms which underlie the pathogenesis of SAMS remain unclear, though there are several identifiable factors that appear to increase the likelihood of its onset, such as female gender, old age, hypothyroidism, lower body mass index (BMI), strenuous exercise, physical disability and low vitamin D levels [11–14]. Pharmacological characteristics of statins themselves, namely lipophilicity and dose, are also postulated to affect the frequency of SAMS; however, data from RCTs regarding these associations is inconsistent [5,15–20].

Several meta-analyses and reviews have investigated the overall effect of statins on the development of adverse muscle symptoms in RCTs [18,21–24]. Unlike these previous studies, however, the present meta-analysis includes results from RCTs which have recruited individuals with a statin intolerance. Indeed, in comparison to past meta-analyses, the inclusion criteria of this investigation is broader with no restrictions placed on sample size, study duration/follow-up period or study quality. Having a broader inclusion criteria allows for a wider demographic of study participants so that unbiased and representative outcomes may be obtained [25]. Furthermore, while previous meta-analyses have considered the effect of lipophilicity and/or dose on the development of SAMS [22,23], the present study has sought to provide a more in-depth analysis of these factors. Namely, statin doses have been standardised in order to account for differences in potency between these medications and the effect this may have on the pathogenesis of SAMS [26,27]. Ultimately, the present systematic review and meta-analysis aimed to update and further the findings of previous meta-analyses by assessing the impact of statin lipophilicity and dose on the frequency of adverse skeletal muscle events across a broader range of participants in order to clarify the relationship between these pharmacological factors and the onset of SAMS.

2. Methods

2.1. Search strategy

This systematic review and meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and the study protocol was registered with International Prospective Register for Systematic Reviews (PROSPERO - CRD42016048342) [28]. PubMed and Scopus databases were searched from inception to 1 June 2017 using a combination of the terms, 'lovastatin', 'fluvastatin', 'pitavastatin', 'simvastatin', 'atorvastatin', 'rosuvastatin', 'pravastatin', 'cerivastatin', 'myalgia', 'statin-induced myopathy' and 'myopathy' (**Supplementary Table S1**). Reference lists of selected articles were also searched to identify further sources.

2.2. Eligibility criteria

All publications included in the review were screened according to selection criteria constructed *a priori*. RCTs (parallel or cross-over design) with at least one group randomised to statin monotherapy as treatment, and a placebo/usual care group as comparator, were included. If RCTs also included a group which had been given statins in combination with other lipid-lowering pharmaceuticals, only the data from the statin monotherapy and control groups were collected. Studies were required to be written in English, include participants $\geq$ 18 years and to have explicitly reported the frequency of at least one type of adverse skeletal muscle event amongst participants. For RCTs with a cross-over design, only events unique to each group (i.e. adverse muscle symptoms with statin but not placebo, or vice versa) were counted. There was no minimum follow-up period, or sample size specifications, however, RCTs were excluded if patients were required to take medications associated with an increased risk of myotoxicity when used in combination with statins (e.g. cyclosporine). Trials which administered vitamin D/coenzyme Q10 supplements to participants were not excluded, but both statin monotherapy and control groups must have received the supplement. Studies were removed if they included participants with other conditions known to cause adverse muscle-related effects (e.g. dengue fever). Duplicate publications and case-control studies were also omitted from the review.

2.3. Data extraction

Titles, abstracts and full articles (if applicable) were screened according to predefined selection criteria. Information pertaining to study type, randomisation methods, blinding, patient characteristics, sample size, interventions, trial duration, low-density lipoprotein cholesterol (LDL-C) entry criteria, primary outcomes, creatine kinase (CK) levels and adverse muscle symptoms was collected. Screening and coding of data was performed independently by two authors. Any discrepancies were resolved through discussion or by the inclusion of a third author.

2.4. Quality of study design and risk of bias assessment

The quality of study design was assessed using the Jadad Quality Scale [29]. Studies were not excluded if they were identified as low quality (Jadad score $\leq$2), but a sensitivity analysis to establish the effect of including these trials was conducted. Publication bias was evaluated for the main analysis using a funnel plot graph and Egger regression asymmetry test [30].

2.5. Data synthesis and analysis

The primary endpoint for the meta-analysis was SAMS (including all incidences of muscle pain/weakness, myositis, myalgia, myopathy and rhabdomyolysis). Elevations in CK ≤10 x the upper limit of normal (ULN) that were unaccompanied by muscle symptoms were not considered to be myotoxic events. The classification of adverse skeletal muscle events used by each trial differed so muscle symptoms were grouped under the term/s used by each study. Furthermore, all reports of muscle-related adverse effects were pooled for the primary analysis in order to account for the variations in classifications.

Comprehensive meta-analysis software version 3 (Biostat, New Jersey) was used for all analyses, all tests were two-sided and a probability level <0.05 was considered significant. For each trial, the summary RR and 95% CI for the primary endpoint were calculated from the number of adverse muscle-related events and total number of participants in the statin and control groups. For multi-arm studies in which participants were treated with either a lipophilic or hydrophilic statin, or randomised to one of multiple doses, data from each intervention group was combined to create a single pairwise comparison, as recommended by the *Cochrane Handbook* [31]. RRs from individual trials were pooled using the DerSimonian-Laird random effect model [32] (including Z-test) and reported using a Forest plot. A risk ratio of less than 1 favoured the statin-treated population. Heterogeneity across the included trials was analysed using the heterogeneity χ^2 (Cochrane Q) test and quantitatively assessed using the I^2 index [33]. For the I^2 statistic, values <30% represented low variation, >30% but <60% indicated moderate variation and >60% signified high variation.

2.6. Sensitivity and subgroup analyses

Sensitivity analyses limited to (i) parallel-design trials, (ii) high quality studies (Jadad score ≥3) or (iii) investigations with sample sizes ≥50 were also performed. Other sensitivity analyses performed were: (i) excluding multi-arm studies, (ii) excluding trials which administered cerivastatin, (iii) excluding studies conducted in Asia (or with predominately Asian study populations), (iv) excluding trials with clinically-based changes in doses (i.e. statin doses changed in response to lipid-lowering targets) and (v) excluding studies which recruited participants with a history of statin intolerance. These assessments were conducted in order to evaluate the impact of each individual characteristic on the primary outcome. The effect of individual studies on the overall results of the meta-analysis were also investigated using the leave-one-out method [30].

Categorical moderator analysis or univariate meta-regression were conducted to investigate the association between statin lipophilicity (see **Supplementary Table S2** for classifications) and dose on the risk of total adverse skeletal muscle events. Doses were standardised to atorvastatin dose equivalents (see **Supplementary Table S2**) and then grouped as low (<40 mg) or high (≥40 mg). For the meta-regression, the natural log-transformed RRs were modelled as a linear function of dose. Multi-arm studies were excluded from their respective lipophilicity and dose moderator analyses. A categorical moderator analysis to assess the combination of statin lipophilicity and dose was also completed with the following classifications: lipophilic + high dose, lipophilic + low dose, hydrophilic + high dose or hydrophilic + low dose. Subgroup analyses on LDL-C entry criteria, myopathy/CK/statin sensitivity exclusion criteria, mean participant age and median follow-up period were performed to determine their impact on the pooled RR. A moderator analysis comparing the incidence of muscle-related adverse events in trials including only female or male participants was also conducted to assess if there was a gender-associated effect on the risk of SAMS. Detailed information on each subgroup comparison is outlined in the appendix (**Supplementary Table S3**).

3. Results

3.1. Characteristics of included studies

Of the RCTs reviewed, 135 fulfilled the inclusion criteria, six were cross-over RCTs and 129 were parallel studies (Fig. 1). Sixteen trials were not double-blinded and 86 stated they were randomised but did not give the specific method of sequence generation. Overall, 121 studies scored a value ≥3 on the Jadad quality assessment scale (**Supplementary Table S4**). In total, 192 977 participants were randomised of whom 100 431 received statin therapy and 92 546 patients were allocated to placebo or usual care. Additional study characteristics are summarised in Table 1 and a full description of baseline parameters for all trials is provided in the appendix (**Supplementary Table S5**).

3.2. Main outcomes

In total, 8 775 statin-treated individuals and 7 885 study participants receiving placebo/usual care were reported to have experienced some form of adverse muscle symptoms (**Supplementary Table S6**). Results from the meta-analysis indicated that statin therapy was associated with a significant increase in the risk of developing adverse skeletal muscle symptoms, but the magnitude of this effect was minimal (RR = 1.050; 95% CI = 1.014–1.089; $P = 0.007$) (Fig. 2). RRs could not be computed for 32 studies as there were no cases of adverse muscle-related events in either the intervention or control groups.

No significant heterogeneity was observed between trials (Cochrane Q = 105.471; $df = 102$; $I^2 = 3.291\%$; $P = 0.387$). The funnel plot of standard error by effect size was asymmetrical (**Supplementary Fig. S1**) and the Egger linear regression test confirmed that there was significant publication bias (intercept = 0.364; standard error = 0.107; 95% CI = 0.150 to 0.577; $P = 0.001$), likely reflecting a disproportionate association of smaller studies with larger effect sizes compared to larger trials. Notably, this result may not reflect a bias toward publishing studies with a significant increase in SAMS as the primary endpoint in nearly all of these RCTs was on cardiovascular outcomes, not adverse effects.

3.3. Sensitivity and subgroup analyses

Excluding studies with a Jadad score ≤2, or sample size <50, did not impact on the overall meta-analysis results (Table 2). Similarly, excluding multi-arm trials, studies which administered cerivastatin or trials conducted in Asia did not significantly affect the pooled RR (Table 2). Excluding studies with changes in dose that were clinically-based (e.g. doses were doubled to increase LDL-C reduction) also did not affect the primary outcome (Table 2).

The *a priori* selection criteria of this meta-analysis allowed for the inclusion of RCTs which recruited participants with a history of statin intolerance. A sensitivity analysis excluding these three trials [34–36] showed that statin therapy was no longer associated with a significant effect on the risk of adverse skeletal muscle effects (Table 2). In particular, the leave-one-out sensitivity analysis identified that the significant result of this meta-analysis was driven by the GAUSS-3 trial with exclusion of this study producing a non-significant result for the primary outcome (RR = 1.020; 95% CI = 0.993–1.048; $P = 0.155$; $I^2 = 0.00$). Additionally, the HOPE-3 trial [37] also contributed to the statistically significant result of this meta-analysis, but its influence on the subgroup analyses was limited (**Supplementary Table S7**).

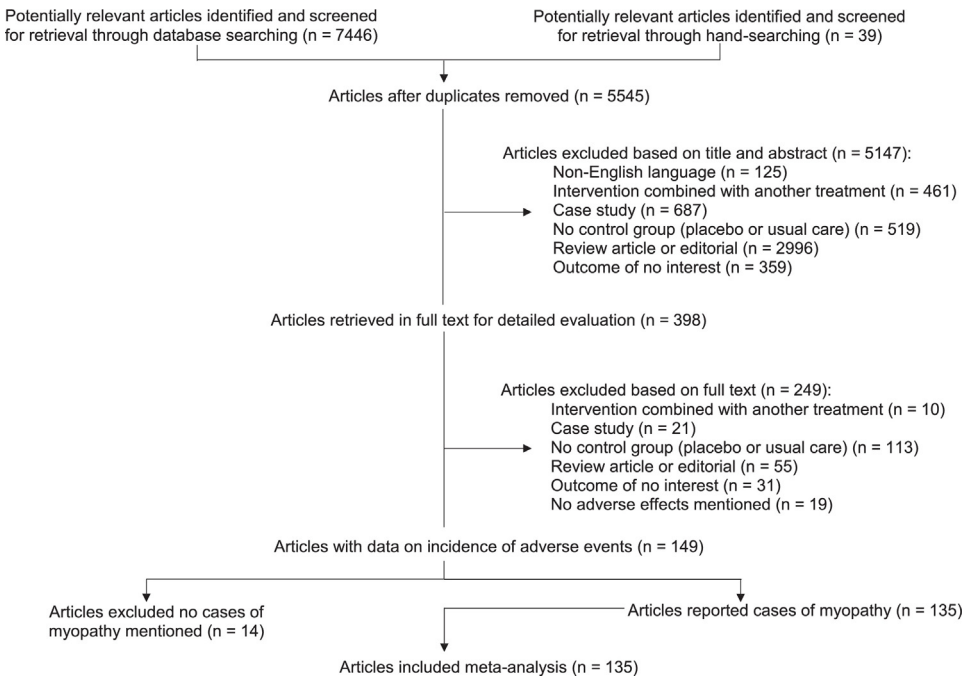

Fig. 1. Flow diagram of systematic search.

Table 1
Characteristics of RCTs included in a meta-analysis of statin lipophilicity, dose and muscle symptoms.

	Average	Range
Number of participants	1430	8–20 536
Follow-up period (y)	1.3	0.04–5.6
Prescribed dose (mg*)	25	0.625–320
	Number of studies	
Prescribed statin:		
Atorvastatin (L)	37	
Cerivastatin (L)	3	
Fluvastatin (L)	9	
Lovastatin (L)	9	
Pitavastatin (L)	1	
Pravastatin (H)	34	
Rosuvastatin (H)	16	
Simvastatin (L)	35	
Recruited individuals with a history of statin intolerance:		
Yes	3	
No	132	
Myopathy/CK/statin sensitivity exclusion criteria:		
Explicit exclusion criteria	47	
No explicit exclusion criteria	88	
Gender:		
Only female participants	3	
Only male participants	9	
Entry LDL-C criteria (mmol L^{-1}):		
$\geq$ 3.4	38	
< 3.4	2	
< 3.4 and $\geq$3.4	21	
Not specified	74	
Study conducted in Asia:		
Yes	5	
No	130	
Clinically-based change in dose:		
Yes	25	
No or not reported	110	

*Normalised to atorvastatin dose equivalents; CK, creatine kinase; H, hydrophilic, L, lipophilic; LDL-C, low-density lipoprotein cholesterol; RCTs, randomised controlled trials; y, years.

Contrastingly, the inclusion of the GAUSS-3 trial impacted considerably on the results of the subgroup and sensitivity analyses. For instance, as GAUSS-3 employed a cross-over study design, it accounted for the different RR observed when the primary analysis was limited to RCTs with a parallel study design (Table 2). The categorical moderator analysis of lipophilicity showed that

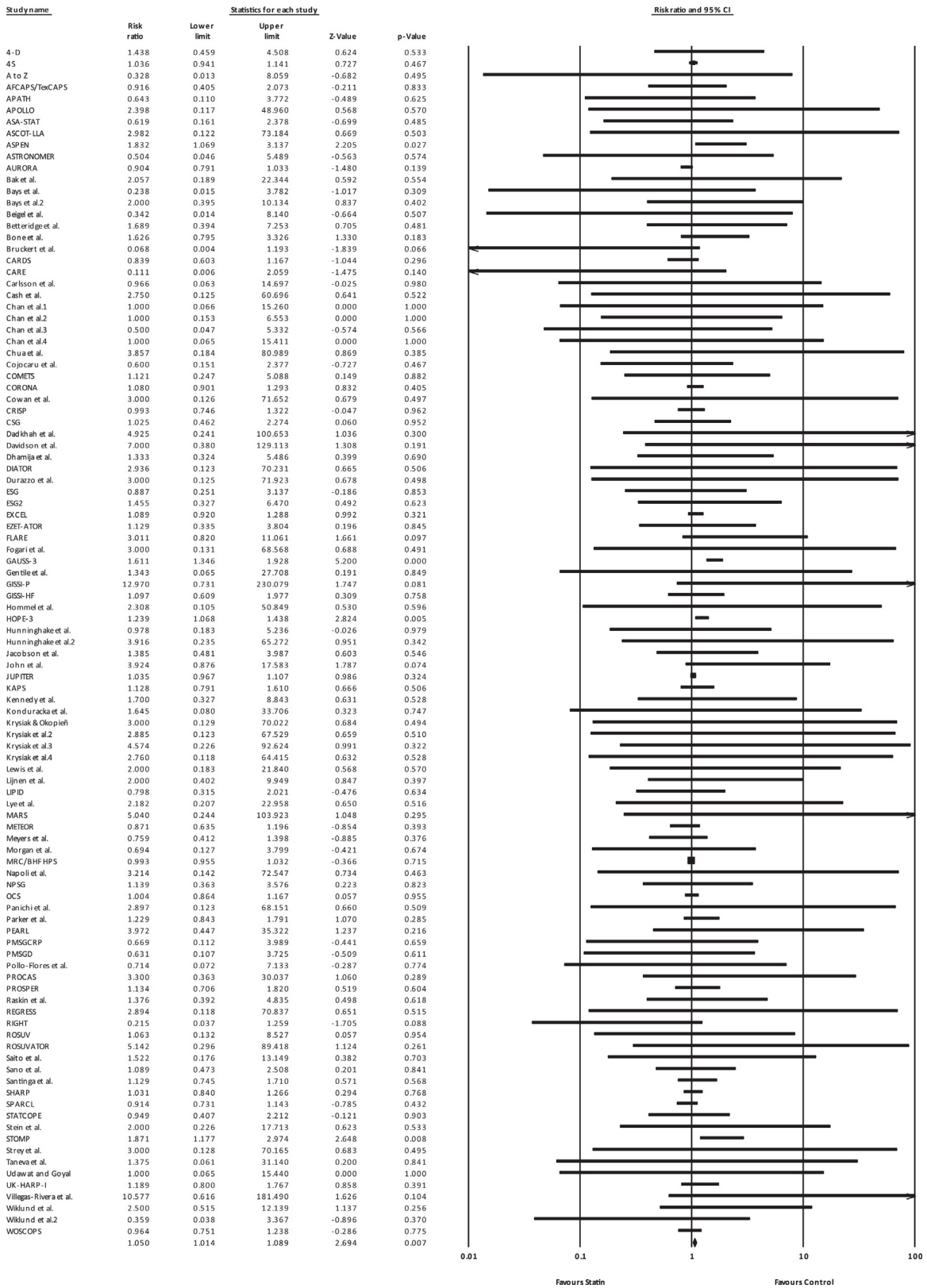

Fig. 2. Forest plot of the meta-analysis of statin use and overall incidence of myopathy. Risk ratios and 95% confidence intervals are displayed. The pooled effect estimate is from a random-effects model. Only data for which risk ratios could be calculated are shown.

lipophilic statins caused a significant increase in the risk of adverse muscle symptoms while hydrophilic statins did not, but the magnitude of this effect was minute (Table 2). When the GAUSS-3 study was excluded from this analysis, however, no significant impact of lipophilic statins on the risk of SAMS was observed. Meta-regression analysis of prescribed doses demonstrated no significant association between dose and the incidence of adverse muscle-related events (slope: −0.0010; 95% CI: −0.0029 to 0.0008;

Table 2
Results of sensitivity and subgroup analyses of RCTs included in a meta-analysis of statin lipophilicity, dose and muscle symptoms.

Sensitivity and subgroup analysis	Risk ratio (95% confidence interval) Risk of muscle symptoms
Sensitivity analysis:	
Parallel design RCTs	1.018 (0.991, 1.047), P = 0.187, n = 97
Jadad score ≥3	1.063 (1.018, 1.110), P = 0.005, n = 94
Sample size ≥50	1.062 (1.016, 1.109), P = 0.007, n = 89
Excluding trials with two or more different statin intervention groups	1.057 (1.016, 1.100), P = 0.006, n = 99
Excluding trials prescribing two or more different statin doses	1.079 (1.019, 1.141), P = 0.009, n = 74
Excluding studies which prescribed cerivastatin	1.049 (1.013, 1.087), P = 0.007, n = 100
Excluding studies conducted in Asia	1.053 (1.014, 1.093), P = 0.007, n = 100
Excluding studies with clinically-based change in dose	1.072 (1.020, 1.126), P = 0.006, n = 83
Excluding studies recruiting participants with previous statin intolerance	1.019 (0.991, 1.047), P = 0.180, n = 100
Subgroup analysis:	
LDL-C entry criteria (mmol L^{-1}):	
> 3.4	1.083 (0.977, 1.200), P = 0.130, n = 30
< 3.4	1.035 (0.967, 1.108), P = 0.317, n = 2
< 3.4 and ≥3.4	1.244 (0.973, 1.591), P = 0.081, n = 17
Not specified	1.011 (0.980, 1.044), P = 0.479, n = 54
Myopathy/CK/statin sensitivity exclusion criteria:	
Explicit criteria	1.004 (0.974, 1.035), P = 0.797, n = 36
No explicit criteria	1.134 (1.070, 1.202), P = < 0.001, n = 67
Lipophilicity:	
Lipophilic	1.101 (1.025, 1.183), P = 0.009, n = 60
Hydrophilic	1.039 (0.988, 1.091), P = 0.134, n = 39
Prescribed dose:	
Low (<40 mg*)	1.106 (1.021, 1.199), P = 0.014, n = 56
High (≥40 mg*)	1.056 (0.958, 1.164), P = 0.275, n = 18
Lipophilicity and dose treatment combination:	
Lipophilic+Low	1.096 (1.014, 1.185), P = 0.021, n = 46
Lipophilic+High	1.345 (0.887, 2.040), P = 0.163, n = 9
Hydrophilic+Low	1.050 (0.904, 1.221), P = 0.521, n = 27
Hydrophilic+High	1.041 (0.938, 1.155), P = 0.454, n = 9
Median follow-up period:	
≥6 m	1.017 (0.990, 1.045), P = 0.229, n = 56
<6 m	1.426 (1.244, 1.634), P = <0.001, n = 47
Mean participant age:	
≥65 y.o	1.072 (1.017, 1.130), P = 0.010, n = 22
<65 y.o	1.076 (1.002, 1.156), P = 0.044, n = 77
Gender:	
Only female participants	1.085 (0.515, 2.285), P = 0.831, n = 2
Only male participants	1.041 (0.852, 1.273), P = 0.693, n = 7

*All doses normalised to atorvastatin dose equivalents; $P < 0.05$ is significant; CK, creatine kinase; LDL-C, low-density lipoprotein cholesterol; m, months; RCTs, randomised controlled trial; y.o, years old.

$P = 0.267$; $R^2 = 0.00$) (Fig. 3). The subgroup analysis of dose, however, showed that low-dose statin therapy produced a significant increase in SAMS risk while high-dose therapy did not (Table 2). Nonetheless, with the exclusion of the GAUSS-3 study, the significant impact of low-dose statin therapy was no longer observed. The subgroup analysis of lipophilicity and dose treatment combinations identified that only the lipophilic + low dose statin combination therapy had a significant, but marginal, impact on the risk of developing adverse skeletal muscle effects. Again, however, it was the GAUSS-3 study which had influenced the significance with removal of this study eliminating this association.

Neither gender nor baseline LDL-C classifications showed a significant impact on risk of developing adverse muscle symptoms in any case (Table 2). Trials with mean follow-up periods of <6 months and no explicit myopathy/CK/statin sensitivity exclusion criteria showed a significant increase in the risk of muscle-related adverse effects with statin use (Table 2). Both mean participant age subgroups (≥65 y.o. or <65 y.o.) had significant associations with SAMS and similar RRs. Following removal of the GAUSS-3 study, however, only the associations for mean age ≥65 y.o. and no myopathy/CK/statin sensitivity exclusion criteria maintained significance (**Supplementary Table S7**). Nonetheless, in each case the actual increase in the risk was limited. Furthermore, the statistical significance of the association between mean age ≥65 y.o. and SAMS was lost following removal of the HOPE-3 study (**Supplementary Table S7**). There was only one trial which prescribed vitamin D supplementation to patients [38], and the leave-one-out sensitivity analysis identified that there was no significant effect of including this study on the meta-analysis results (RR = 1.047; 95% CI = 1.012–1.085; $P = 0.009$).

In addition to re-conducting the meta-analysis following exclusion of the GAUSS-3 study, a winsorised analysis of the results was also performed (see **Supplementary Table S8** for description and results). Winsorising is a method of replacing the result of a study deemed to be an outlier with the next similar outcome in the same category of study design and setting to moderate the effect of extreme values [39]. Results for both the winsorised data set and analyses following exclusion of GAUSS-3 were similar. There was a slight difference in the outcome of the mean follow-up period subgroup analysis with the association between adverse muscle symptoms and a mean study duration <6 months maintaining significance for the winsorised results. Nonetheless, the calculated RRs between the different data sets were still analogous.

4. Discussion

This systematic review and meta-analysis of 135 RCTs found that statin therapy produced a 1.050-fold increase in the risk of adverse muscle symptoms. That is, approximately 105 statin-treated participants experienced SAMS for every 100 placebo-treated individuals presenting with muscle complaints. Ultimately, this result demonstrates a limited impact of statins on adverse skeletal muscle

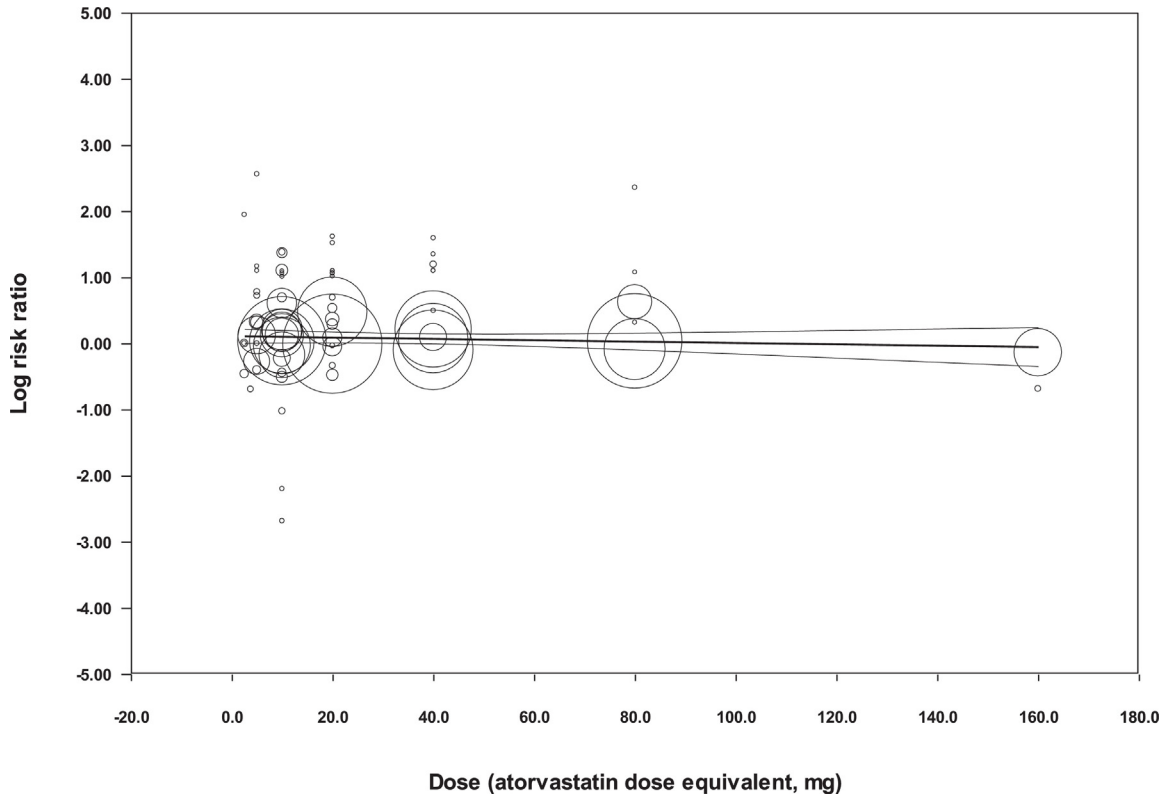

Fig. 3. Risk ratios of myopathy associated with statin use as a function of dose (mg). Risk ratios are displayed on a logarithmic scale. Linear regression line is accompanied by the upper and lower bounds for the 95% confidence intervals (curving lines). Circle shapes represent the individual studies. Only data for which risk ratios could be calculated are shown.

events in RCTs, which is consistent with other meta-analyses and reviews [18,21–24]. Nonetheless, unlike these previous works, the RR calculated in this meta-analysis was statistically significant. Sensitivity analyses revealed that a major cause of this significance was the inclusion of RCTs which recruited participants with a history of statin intolerance, especially the GAUSS-3 study [34], with exclusion of this trial producing a non-significant RR. The *a priori* selection criteria of this study did not exclude studies recruiting individuals with previous statin intolerance as this meta-analysis aimed to use a broad range of RCTs, and thus, participants. Furthermore, as the GAUSS-3 trial was published only recently (2016), it has not been included in previous meta-analyses of statin RCTs [18,21–24].

The GAUSS-3 study itself involved two stages: (i) Phase A in which patients with a history of statin intolerance were assigned to atorvastatin or placebo treatment for 10 weeks and (ii) Phase B in which individuals who experience muscle-related symptoms with atorvastatin alone (or those with a documented history of CK elevation >10 × the upper limit of normal accompanied by muscle symptoms while on statin therapy) received either evolocumab or ezetimibe for 24 weeks [34]. Only data from Phase A was suitable for inclusion in this meta-analysis. Unlike the other RCTs investigated in this meta-analysis, the GAUSS-3 study was designed such that only those patients with reproducible SAMS were entered into Phase B of the study. Accordingly, it can be speculated that, comparatively, a greater emphasis was placed on the emergence of adverse muscle events in GAUSS-3 in contrast to other statin RCTs which focussed more heavily on cardiovascular outcomes (**Supplementary Table S5**). Hence, the combination of statin intolerant participants, and greater attention on the incidence of SAMS, may account for the stronger association between statin use and muscle-related adverse events observed in the GAUSS-3 trial, and its consequent influence on the results of this meta-analysis.

The observation that a history of statin intolerance can increase the likelihood of SAMS is not unexpected. Indeed, RCTs tend to exclude such individuals from statin trials as they are likely to drop-out post-randomisation due to adverse effects [40,41]. Accordingly, a subgroup analysis identified that those studies which did not explicitly exclude individuals based on predefined myopathy/CK/statin sensitivity entry criteria were associated with a significant increase in the risk of SAMS. This result was maintained even when GAUSS-3, and other trials including participants with a known history of statin intolerance, were removed from the analysis (data not shown). Nonetheless, while the difference in risk between placebo and statin-treated participants was statistically significant, the overall increase in the frequency of adverse muscle symptoms was marginal. Hence, the clinical impact of a history of statin intolerance/myopathy on the risk of SAMS in practice could be quite modest.

The primary goal of this meta-analysis was to clarify the impact of statin lipophilicity, and dose, on the frequency of muscle-related adverse effects in a large pool of RCTs. Increased myotoxicity with lipophilic statins has been attributed to their ability to non-selectively diffuse across the cell membranes of extra-hepatic tissues (including the skeletal muscle), even though some have lower systemic bioavailability compared to hydrophilic formulations (**Supplementary Table S2**) [22,42–44]. With exclusion of the GAUSS-3 study, however, this meta-analysis found no significant association between either lipophilic or hydrophilic statins and the risk of adverse skeletal muscle events. Moreover, even when GAUSS-3 was included, the actual increase in the risk of SAMS with lipophilic statins was minute. A meta-analysis of statin RCTs recruiting individuals ≥65 y.o. also found limited evidence that lipophilic statins were associated with a greater risk of SAMS [22]. Thus, although a physiological effect of lipophilicity on statin-associated myotoxicity has been demonstrated in preclinical

studies [42,43,45], the findings of this meta-analysis indicate that the actual biological impact of lipophilicity alone on SAMS may be negligible.

This point is further demonstrated by the sensitivity analysis excluding studies using cerivastatin, the most lipophilic of all statins, which showed no effect on the pooled effect size. Cerivastatin was removed from the market in 2001 due to its association with 52 patient deaths from rhabdomyolysis with acute renal failure, 50% of which occurred with a cerivastatin-gemfibrozil combination therapy [46]. No cases of rhabdomyolysis were reported in any of the RCTs using cerivastatin [47–49], possibly because these trials did not co-administer concomitant medications, such as gemfibrozil [50]. Indeed, the increased risk of SAMS with lipophilic statins typically observed in clinical practice may not be due to the actual lipophilicity of these drugs, but rather because lipophilic statins tend to have more drug–drug interactions compared to their hydrophilic counterparts [14]. While the use of polypharmacy and concomitant medications can be largely controlled in RCTs, this is not always the case in the clinical setting so the incidence of SAMS with lipophilic statins can be higher [51].

A dose-dependent nature of SAMS has been demonstrated by several RCTs, as well as in clinical practice [12,20,52]. A higher dose treatment regimen can cause higher plasma concentrations of statins, which in turn, is associated with a greater likelihood of myotoxicity [53,54]. The present meta-analysis, however, did not find a dose-dependent association between statins and the risk of muscle-related adverse effects. The lack of a relationship between high-doses and SAMS is contrary to most reports, however, this is not the first study to report an absence of this effect. Indeed, the Treating to New Targets (TNT) study, which included 10 001 patients, also found no difference in the rate of statin myalgia between high-dose versus low-dose atorvastatin treatment regimens [55]. Likewise, a recent meta-analysis by Naci et al. [23] found no apparent dose-response relationship for myalgia, however this observation was made using unstandardised doses. The present meta-analysis has demonstrated that even when statin dose equivalents are used to account for differences in drug potencies, there is little impact of dose on the frequency of SAMS in RCTs. Thus, rather than being a generalisable effect, these results indicate that the potential association between dose and the risk of SAMS is dependent on the individual. Indeed, persons with a history of statin intolerance are known to develop SAMS at low-dose prescriptions that are otherwise well-tolerated by the majority of statin-treated patients [56]. It seems more likely that high dose statin therapy has a 'synergistic' effect with other risk factors of SAMS (e.g. polypharmacy, genetic factors, family history of muscle disorders) to cause myotoxicity rather than being a sole driver of skeletal muscle damage. Thus, akin to lipophilicity, statin dose alone may not be a major contributor to the development of SAMS in RCTs.

In addition to lipophilicity and dose, other factors, including female gender, are postulated to influence the rate of SAMS [11–14]. In this meta-analysis, however, no significant association was observed between female-only studies and the frequency of SAMS. Not all studies have associated female gender with an increased risk of adverse muscle symptoms [57], however the majority have done so, making the findings of this meta-analysis contrary to these reports [58,59]. Hence, in light of this result, as well the current ambiguity surrounding the mechanism underlying the increased sensitivity of females to SAMS [59], further investigation into this relationship is required.

Persons of Asian ancestry have been suggested to be predisposed to SAMS as altered metabolism and clearance of statins in these individuals can result in higher plasma statin concentrations [60,61]. A sensitivity analysis excluding studies recruiting Asian participants, however, showed no change in the pooled effect size which casts uncertainty on this relationship. Similarly, a recent retrospective cohort study of older adults reported no difference in the risk of statin-related myotoxicity in Chinese compared to non-Chinese participants [60].

Older age has also been shown to be a risk factor of SAMS [12,62]. While the initial outcome of this meta-analysis showed that both mean participant age subgroups (≥65 y.o. or <65 y.o.) had significant associations with SAMS, exclusion of the GAUSS-3 study resulted in only RCTs with a mean age of ≥65 y.o maintaining a significant effect. Although this finding supports previous observations, the actual RR indicated that the impact of older age on the risk of SAMS was minimal. Furthermore, the statistical significance of this association was lost if the HOPE-3 study was removed from the analysis. The strong relationship between statin therapy and adverse muscle-related events observed in the HOPE-3 trial may indeed be due to the fact that this investigation only included men ≥55 y.o. or women ≥60 y.o. [37] Nonetheless, other studies, such as PROSPER [63] and JUPITER [64], also recruited only older participants and yet no significant associations between statins and adverse muscle symptoms were found in these trials (Fig. 2). The lack of sufficient evidence to support an age-associated effect of SAMS means further studies on the impact of age on statin myotoxicity would be beneficial. Indeed, the higher incidence of comorbidities, and thus polypharmacy to treat these conditions, may be the cause of the increased occurrence of SAMS generally observed in these individuals in clinical practice rather than an age-related physiological changes in skeletal muscle [65].

Another characteristic investigated in this meta-analysis was the effect of baseline LDL-C levels on the risk of SAMS. None of the baseline LDL-C entry criteria subgroups showed a significant association with adverse muscle symptoms. Similarly, Naci et al. [23] found no association between LDL-C levels and adverse events in their assessment of statin-induced side effects. Thus, the incidence of SAMS is likely to be independent of baseline LDL-C levels.

The impact of median follow-up time on the incidence of SAMS was also evaluated. RCTs with a median follow-up period <6 months showed the greatest association between statin therapy and adverse skeletal muscle events. This significance was lost following exclusion of the GAUSS-3 trial (which had a follow-up time of 10 weeks) but was maintained using the winsorised data set. Nonetheless, overall the increase in the risk of SAMS in RCTs with study durations <6 months was still limited. The lack of a noteworthy association between follow-up time and the frequency of muscle-related adverse effects may reflect the fact that SAMS can develop at any time following initiation of statin therapy [63]. The differential time of onset of SAMS adds complexity for physicians when diagnosing this condition in the clinical setting [14].

It has been argued that RCTs cannot provide as useful information on the adverse effects of statins compared to observational studies due to their stringent exclusion criteria [66,67]. Nonetheless, meta-analyses of RCTs remain the 'gold-standard' for drug evaluation [68]. Furthermore, Collins et al. [25] has suggested that meta-analyses of statin RCTs with varied eligibility criteria, and thus different types of participants (age, gender, etc.), can indeed produce unbiased and representative outcomes. Accordingly, this meta-analysis has sought to achieve this by including a large number of participants from various RCTs. Moreover, a further strength of this meta-analysis is the low heterogeneity observed, despite the inclusion of such a large pool of RCTs.

Although this meta-analysis attained its goal of including a broad range of participants, it does have its limitations. The inclusion of trials that recruited individuals with previous statin intolerance (particularly GAUSS-3) was a limitation of this study; however, incorporating such studies did allow for the effect of including statin intolerant individuals in statin RCTs to be evaluated. Likewise, the inclusion of small, and 'low-quality' studies (Jadad score ≤2) may also be a limitation of this meta-analysis, as

evidenced by the funnel plot graph. Nonetheless, a sensitivity analysis excluding studies with population sizes <50 persons, or Jadad quality score ≤2, produced no appreciable changes in the results. The inclusion of these additional studies can thus be considered as a benefit for this meta-analysis as they have assisted in providing an unbiased presentation of the risk of SAMS in RCTs.

One drawback of this meta-analysis was the absence of analyses assessing the influence of BMI and physical exercise on the frequency of adverse muscle symptoms in statin RCTs. Both of these factors are reported to increase the risk of SAMS [12,14]; however, the assessment and/or reporting of these parameters in statin RCTs is limited. For instance, only three of the 135 RCTs included in this meta-analysis reported on the degree of physical exercise undertaken by participants during the study. Similarly, only 81 trials (60%) reported BMI data. A meta-regression on the available data demonstrated no significant association between mean BMI and the risk of SAMS (data not shown).

A moderator analysis to assess the potential association between medication adherence and the primary outcome was also unable to be conducted. A lack of compliance with drug treatments may have influenced the results of the meta-analysis, however, only 45% of the included RCTs explicitly reported data on medication adherence. Akin to BMI, a meta-regression using the available data showed no significant relationship between percentage medication adherence and the risk of adverse muscle symptoms (data not shown).

5. Conclusions

Ultimately, the pooled results form 135 RCTs in this meta-analysis indicate that there is a limited effect of statin therapy on muscle-related adverse effects in RCTs, even in those individuals with a history of myopathy/CK/statin intolerance. The results of this study show that neither statin lipophilicity, nor dose, have a clinically significant impact on the development of SAMS. There was also no significant effect of gender, ethnicity, baseline LDL-C levels or median follow-up time on the incidence of adverse statin-induced skeletal muscle effects, though a limited age-associated effect may be plausible. Accordingly, further investigation into the factors which influence the development of SAMS is required in order to improve patient compliance with these pharmaceuticals.

6. Funding

The researchers gratefully acknowledge the financial support provided by the Australian Government's Research Training Program. The funding source had no involvement in: the study design; collection, analysis or interpretation of data; the writing of the manuscript or in the decision to submit this work for publication.

7. Conflicts of interest

None.

Acknowledgements

All contributors to the manuscript are listed as authors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2017.09.013.

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CHAPTER 3

Validation of a clinically-relevant rodent model of statin-associated muscle symptoms for use in pharmacological studies

Preamble

The results presented Chapter 2 demonstrated that there remains considerable ambiguity in the scientific literature concerning the role of statin dose and lipophilicity in the pathogenesis of SAMS. As noted in the preceding chapter, this uncertainty may relate to the influence of the nocebo effect, polypharmacy and/or comorbidities on reported rates of SAMS. In animal studies, these variables are eliminated; thus, a clearer understanding of the aetiology of SAMS can be obtained. Moreover, it is possible to perform more intricate assessments of skeletal muscle integrity using animal studies. Accordingly, rodent-based investigations were completed during this project.

In order to ensure that the data generated from these studies was accurate and clinically-relevant, a suitable model of SAMS had to be identified. Specifically, this treatment protocol needed to reproducibly induce changes in skeletal muscle physiology which were characteristic of SAMS in humans. In order to validate a dosing regimen which met these requirements, a head-to-head comparison study of two established models was conducted. Considerable differences were observed between the physiological alterations induced by each treatment protocol. This variation in effects justified validating the rodent model of SAMS to be used in this project. An appropriate treatment regimen was identified, and this

was used as the platform for assessing the pathogenesis of statin-induced myotoxicity in the subsequent studies of this project.

This chapter contains the manuscript titled, “Validation of a clinically-relevant rodent model of statin-associated muscle symptoms for use in pharmacological studies”, which has been published in *Toxicology and Applied Pharmacology* (2018;360;78-87).

Declaration of co-authorship and contribution

Full bibliographic details and Status

Irwin JC, Fenning AS, Ryan KR & Vella RK. Validation of a clinically-relevant rodent model of statin-associated muscle symptoms for use in pharmacological studies. Toxicol Appl Pharmacol. 2018;360;78-87.
Published

Nature of Candidate's Contribution, including percentage of total

JC Irwin (80%): Study conceptualisation and design, acquisition of data, analysis and interpretation of data, writing of original draft, critical revision of manuscript and approval of final version.

Nature of all Co-Authors' Contributions, including percentage of total

AS Fenning (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version. KR Ryan (5%): Acquisition of data, critical revision of manuscript and approval of final version. RK Vella (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Toxicology and Applied Pharmacology 360 (2018) 78–87

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Validation of a clinically-relevant rodent model of statin-associated muscle symptoms for use in pharmacological studies

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ARTICLE INFO

Keywords:
Rodent model
Statin-associated muscle symptoms
Simvastatin

ABSTRACT

Various rodent models of statin-associated muscle symptoms (SAMS) have been used to investigate the aetiology of statin myotoxicity. Variability between these models, however, may be contributing to the ambiguity currently surrounding the pathogenesis of SAMS. Furthermore, few studies have assessed the reproducibility of these models. The aim of this study was to compare two established rodent models of statin myotoxicity, differing in treatment duration and dose, to determine which reproducibly caused changes characteristic of SAMS. Isolated skeletal muscle organ bath experiments, biochemical analyses, real-time quantitative-PCR and biometric assessments were used to compare changes in skeletal muscle and renal integrity in statin-treated animals and time-matched control groups. The SIM80 model (80 mg kg^{-1} day^{-1} simvastatin for 14 days) produced fibre-selective skeletal muscle damage characteristic of SAMS. Indeed, fast-twitch gastrocnemius muscles showed increased *Atrogin-1* expression, reduced peak force of contraction and decreased *Myh2* expression while slow-twitch soleus muscles were unaffected. Contrastingly, the SIM50 model (50 mg kg^{-1} day^{-1} simvastatin for 30 days) produced little evidence of significant skeletal muscle damage. Neither statin treatment protocol caused significant pathological changes to the kidney. The results of this study indicate that the SIM80 model induces a type of SAMS in rodents that resembles the presentation of statin-induced myalgia in humans. The findings support that the SIM80 model is reproducible and can thus be reliably used as a platform to assess the aetiology and treatment of this condition.

1. Introduction

Statin therapy is pivotal for the primary and secondary prevention of cardiovascular disease (CVD), particularly in individuals with coronary heart disease, diabetes or a history of stroke or myocardial infarction (Heller et al., 2017; Maningat et al., 2012). While generally well-tolerated, approximately 10–25% of individuals taking statins experience adverse statin-associated muscle symptoms (SAMS) (Khan et al., 2015). The clinical manifestation of SAMS varies considerably and may present as myalgia (muscle pain/cramps with normal serum creatine kinase, CK), myopathy (muscle weakness with normal or elevated CK), myositis (muscle inflammation with elevated CK) or myonecrosis/rhabdomyolysis (hyperCKemia with/without myoglobinuria or acute renal failure) (Rosenson et al., 2014; Muntean et al., 2017). The onset of SAMS is clinically significant as it can reduce quality of life in affected individuals (Parker and Thompson, 2012). Moreover, its development is one of the main contributors to statin discontinuation (Maningat et al., 2012), which in turn, is associated with a higher risk of mortality from CVD (Banach et al., 2015; Toth et al., 2018; Tziomalos et al., 2008).

The lack of consensus on the exact mechanism which underlies SAMS presents a considerable obstacle for effectively managing these events and thus improving cardiovascular health (Moßhammer et al., 2014; Taha et al., 2016; Irwin et al., 2018). Extensive rodent studies have been performed to elucidate the pathophysiology of statin myotoxicity using a range of statins including: simvastatin (Westwood et al., 2005; Simsek Ozek et al., 2014; Mallinson et al., 2009; Mallinson et al., 2012; Pierno et al., 1999; Goodman et al., 2015; Reijneveld et al., 1996; Sidaway et al., 2009), cerivastatin (Westwood et al., 2005; Sidaway et al., 2009; Obayashi et al., 2011; Schaefer et al., 2004), atorvastatin

Abbreviations: Ca^{2+}, calcium; CVD, cardiovascular disease; CK, creatine kinase; CON, control; FFCs, force-frequency curves; GAS, gastrocnemius; LDH, lactate dehydrogenase; MG, myoglobin; MTD, maximum tolerable dose; SAMS, statin-associated muscle symptoms; SIM, simvastatin-treated; SOL, soleus; TA, tibialis anterior

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https://doi.org/10.1016/j.taap.2018.09.040
Received 22 June 2018; Received in revised form 8 September 2018; Accepted 26 September 2018
Available online 28 September 2018
0041-008X/

(Camerino et al., 2011; D'Antona et al., 2013; Muraki et al., 2012; El-Ganainy et al., 2016a; El-Ganainy et al., 2016b), lovastatin (Reijneveld et al., 1996; Smith et al., 1991), fluvastatin (Camerino et al., 2011; Sugatani et al., 2010), pravastatin (Reijneveld et al., 1996; Muraki et al., 2012; Smith et al., 1991; Naba et al., 2004) and rosuvastatin (Sidaway et al., 2009; El-Ganainy et al., 2016b; Westwood et al., 2008). In addition to differences in statin type, the age and gender of rats, as well as drug doses, used in these investigations have also varied considerably and each of these variables can influence the severity of SAMS (Irwin et al., 2018; Buettner and Lecker, 2008; Shear et al., 1992). Indeed, male rodents have been used previously in studies of SAMS despite evidence that, in both humans and rodents, females are more sensitive to statin myotoxicity (Schaefer et al., 2004; Sathasivam and Lecky, 2008; Katz et al., 2014; Seachrist et al., 2005). Furthermore, as rodents develop pathological alterations in skeletal muscle with age (Caccia et al., 1979; Altun et al., 2010), it is more appropriate for young rats to be used in models of SAMS to ensure that only statin-associated skeletal muscle damage is assessed.

The differences between rodent models of SAMS are likely to be introducing inconsistencies and/or ambiguity about the exact pathogenesis of this condition. Therefore, in order to produce more consistent findings, a standardised and reproducible rodent model of SAMS is required. This model should induce changes characteristic of SAMS including reduced power output (Mallinson et al., 2015), increased mitochondrial oxidative stress (Bouitbir et al., 2016) and enhanced expression of atrophy-related genes (Hanai et al., 2007). Furthermore, these effects should be fibre-selective whereby type II fast-twitch glycolytic fibres are susceptible to myotoxicity while type I slow-twitch oxidative fibres are resistant to any statin-induced damage (Seachrist et al., 2005; London et al., 1991).

Since the discontinuation of cerivastatin, simvastatin has become the formulation most frequently associated with adverse muscle effects in the clinical setting (Keltz et al., 2013). Simvastatin is also the most commonly used statin in rodent models of SAMS, even though the changes in muscle physiology exerted by statins are similar between the different formulations (Thompson et al., 2016; Gluba-Brzozka et al., 2016). Of the simvastatin treatment schedules employed in rodent studies of SAMS, the most frequently used is 80 mg kg^{-1} day^{-1} for approximately two weeks (Westwood et al., 2005; Mallinson et al., 2009; Goodman et al., 2015; Sidaway et al., 2009). While this high-dose protocol produces a rapid onset of myotoxicity (i.e. within 14 days), it does reach the maximum tolerable dose (MTD) of statins in rodents (Westwood et al., 2005). Hence, there is a risk when using this model that any physiological changes associated with statin myotoxicity may be masked by potential toxicological consequences caused by statin overdose. In light of this, alternative models which administer a lower (mid-range) dose of statin for a longer period, such as 50 mg kg^{-1} day^{-1} for 30 days (Simsek Ozek et al., 2014), may be more preferable for studying SAMS. However, the ability of simvastatin when administered at doses lower than 80 mg kg^{-1} to reproducibly induce noteworthy changes in skeletal muscle functionality has been questioned (Westwood et al., 2005). Moreover, studies in *mdx* dystrophic mice have shown that long-term, low-dose simvastatin treatment (5–10 mg kg^{-1} day^{-1} for 8 months) can actually improve muscle health (Whitehead et al., 2015).

Notably, both treatment protocols use high doses of statins when compared to the amounts prescribed in humans. Indeed, while the average statin dose for humans ranges between 0.1 and 1 mg kg^{-1}, most rodent studies employ concentrations between 1 and 100 mg kg^{-1} (Björkhem-Bergman et al., 2011). The discrepancy in dosages is the result of the pharmacodynamic-resistance to statins displayed by rodents (Westwood et al., 2005; Björkhem-Bergman et al., 2011). Hence, higher doses need to be given to rodents in order to induce the same physiological changes observed in humans at comparatively lower doses (Westwood et al., 2005).

The aim of this investigation was to clarify which of the two aforementioned treatment protocols (two weeks of high-dose treatment or four weeks of mid-range therapy) reproducibly caused changes characteristic of statin myotoxicity in humans when administered to young, female rodents. It is anticipated that the selected model will provide a stable platform against which factors which are postulated to influence the severity or likelihood of SAMS (such as lipophilicity, dose, polypharmacy, gender and age) (Banach et al., 2015; Gluba-Brzozka et al., 2016) can be assessed in order to verify their effects on statin myotoxicity. Likewise, the model may also be used to determine which molecular/metabolic pathways should be targeted to provide novel therapeutic interventions to more effectively manage SAMS.

2. Materials and methods

2.1. Animals and treatment protocols

Young (12-week old) female Wistar rats (250–350 g) were randomised to one of four treatment groups: no intervention for 14 days (CON80), 80 mg kg^{-1} day^{-1} simvastatin for 14 days (SIM80), no interventions for 30 days (CON50) or 50 mg kg^{-1} day^{-1} simvastatin for 30 days (SIM50). Simvastatin was dissolved in solution comprised of 10% v/v Tween 20 in milliQ water as this has been identified as a suitable vehicle to use in pharmacological studies for administering drugs (AL-Wajeeh et al., 2017; Hajrezaie et al., 2012; Porwal et al., 2017; Saeed Al-Wajeeh et al., 2016), including statins (Loch et al., 2006). An a priori power analysis was performed to determine the minimum number of animals required to achieve statistically valid results (alpha level 0.05, power beta level of 0.8, effect size 0.980). Rodents were housed in a constant 12-h light/darkness cycle at a temperature of 22 ± 2 °C and permitted access to water and food (standard rat chow) ad libitum. Experimental procedures were approved by the Animal Ethics Committee of Central Queensland University (CQU AEC 0000019911) under guidelines from the National Medical Research Council of Australia.

2.2. Biometric assessments

Water consumption and changes in body mass was assessed every two or three days during the treatment period, respectively. Upon completion of the dosing protocol, rats were euthanised via a 1.0 mL intraperitoneal injection of sodium pentobarbitone (187.5 mg mL^{-1}) and death was confirmed by a lack of pedal reflex and corneal reflexes. The wet mass of the gastrocnemius (GAS) muscle (predominate fibre type IIB), soleus (SOL) muscle (fibre type I) and tibialis anterior (TA) muscle (fibre type I, IIA and IIB), as normalised to body mass, were recorded. As skeletal muscle injury can result in kidney damage through release of toxic concentrations of myoglobin (Keltz et al., 2013), kidney mass was measured as one indicator of renal health.

2.3. Ex vivo skeletal muscle functional assessment

Isolated skeletal muscle tissue bath experiments were completed using a modified protocol (Simsek Ozek et al., 2014). GAS, SOL and TA muscles were transferred to 25 mL warmed (37 °C) organ baths containing gassed (carbon dioxide (CO_2) 5% / oxygen (O_2) 95%) Krebs-Henseleit buffer (KHB) (all in mM concentrations: sodium chloride 135, potassium chloride 5, magnesium chloride 1, disodium hydrogen phosphate 1, sodium bicarbonate 15, calcium chloride 2 and glucose 1; pH ~ 7.4). Muscles were suspended between two platinum zigzag electrodes and loaded with 2 g tension. Following a 10-min equilibration period, electrical field simulation trains were applied at 100 V at an increasing frequency from 1 to 100 Hz for 5 s every 135 s. Stimulations were induced twice at each frequency; any tissue responses were detected using Grass FT03 transducers and recorded using Lab Chart software and PowerLab® data acquisition units (ADInstruments, Bella Vista, Australia). For SOL and TA muscles, the protocol was halted after

40 and 70 Hz, respectively, as preliminary data showed that no further muscle contraction was induced at higher frequencies in these muscles (data not shown). When constructing the force-frequency curves (FFCs), force responses were normalised to skeletal muscle mass and length using the following formula: force (g)/cm^2 = [force (g) × 1.06 g/cm^3 (specific density of skeletal muscle) × length of muscle (cm)] / mass of muscle (g) (Simsek Ozek et al., 2014).

2.4. Assessment of mRNA expression

Differences in mRNA expression were determined by real-time quantitative-PCR. mRNA was extracted from tissue homogenates using the phenol-chloroform method (Sambrook and Russell, 2006) and evaluated using a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesised using Superscript III reverse transcriptase according to the manufacturer's instructions (Applied Biosystems Inc.). PCR reactions were run using Rotor-Gene Q equipment (Qiagen), Taqman universal PCR master mix and the following Taqman gene expression assays: rat *Gapdh* (Rn99999916_s1), rat *Atrogin-1/Fbxo32* (Rn00591730_m1), rat *Sod2* (Rn00690588_g1), rat *Myh2* (Rn014707656_m1), rat *Myh7* (Rn01488777_g1), rat *Sod1* (Rn00566938_m1), rat *Havcr1/Kim1* (Rn00597703_m1) and rat *Gpx1* (Rn005777994_g1). Relative expression for Taqman-analysed transcripts was calculated using the delta-delta Ct method (Livak and Schmittgen, 2001) and converted to $\log_2$ fold changes for data presentation.

2.5. Biochemical analyses

Blood samples were collected from the abdominal vena cava, centrifuged and stored at −80 °C until the time of analysis. To assess the degree of skeletal muscle damage, serum concentrations of creatine kinase (CK), myoglobin (MG), lactate dehydrogenase (LDH) and calcium (Ca^{2+}) were quantified using a Roche Diagnostics 400 plus Biochemical Analyser and serum levels of urea and uric acid were measured to evaluate renal damage.

Collagen deposition in the renal cortex was measured using a modified colourimetric hydroxyproline assay (Reddy and Enwemeka, 1996). Briefly, samples were homogenised in milli-Q water and placed into Eppendorf tubes spiked with hydroxyproline stock solution. Following hydrolysation with sodium hydroxide (NaOH) at 110 °C, all samples and standards were oxidised with chloramine-T reagent at room temperature. Aliquots were transferred to separate wells of a 96-well plate and incubated at 65 °C with Ehrlich's aldehyde reagent. All samples were performed in duplicate and absorbance was read at 540 nm. Hydroxyproline levels in skeletal muscle were not quantified as statin treatment has been noted to cause only a sporadic increase in collagen fibrils in muscle samples (Schaefer et al., 2004).

2.6. Statistical analysis

Statistical analyses were conducted between each model and its respective control (i.e. CON80 versus SIM80 and CON50 versus SIM50). Normality testing was conducted prior to statistical analyses via the D'Agostino & Pearson test and/or through inspection of histograms. Differences in biometric and biochemical parameters, as well as maximal skeletal muscle force production, were evaluated using independent samples *t*-test with Bonferroni correction for multiple comparisons. Differences in FFCs, water intake and body mass were assessed using repeated-measures two-way ANOVA with Bonferroni post-hoc comparison. Non-linear regression of skeletal muscle FFCs was used to calculate the effective frequency needed to reach 50% of the maximal contraction of the skeletal muscle (EF50) and group values were compared using independent samples *t*-test with Bonferroni post-hoc testing when appropriate. Statistical analysis of mRNA expression was performed using the Mann-Whitney *U* test on the delta Ct values. All data is presented as means with standard deviations, aside from mRNA expression data which is expressed as means with 95% confidence intervals. Statistical significance was set at an alpha level of 0.05 and all analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, USA).

2.7. Reagents and chemicals

Pharmaceutical-grade simvastatin was purchased from the Rockhampton Base Hospital (Australia). Reagents and chemicals for biochemical analyses were of analytical grade and purchased from Sigma Aldrich (Australia) and Thermofisher Scientific (Australia). Reagents for the Cobas Integra® 400 Plus Biochemical Analyser were purchased from Roche Diagnostics (Australia). Unless otherwise stated, solutions were prepared using milli-Q water.

3. Results

3.1. Ex vivo skeletal muscle functional assessment

Main effects analyses of GAS FFCs showed no significant differences for either pairwise comparison (Fig. 1 (a) and (b)). However, there was a significant interaction between frequency and force for the SIM80 versus CON80 analysis so simple effects were measured. These results identified significant differences in force production at 90 and 100 Hz ($P < .05$). Accordingly, analysis of maximum contraction identified that peak force was significantly lower in SIM80 rats compared to CON80 animals ($P < .05$) (Table 1). Furthermore, Log_{10}EF50 was significantly reduced by SIM80 treatment ($P < .05$) (Table 1). Contrastingly, there were no significant differences in peak maximal contraction or Log_{10}EF50 values between the SIM50 and CON50 animals (Table 1). For both the SOL and TA muscles, no significant differences in FFCs or maximum force were observed (Fig. 1 (c) and (d)). There were also no significant differences in Log_{10}EF50 values between SIM80 and CON80 rats (Table 1). Comparison of SIM50 and CON50 groups identified that Log_{10}EF50 values for the SOL were significantly lower in SIM50 rats ($P < .05$), but the opposite was observed for the TA ($P < .05$) (Table 1).

3.2. Assessment of mRNA expression

Atrogin-1 mRNA expression was significantly increased in GAS muscles isolated from SIM80 rats compared to CON80 animals ($P < .05$) (Fig. 2). There was also a significant increase in *Sod2* mRNA levels in the SIM80 group but *Myh2* expression was significantly reduced compared to CON80 animals ($P < .05$) (Fig. 2). No significant differences in *Atrogin-1* or *Myh2* levels were observed in SIM50 rats, however, *Sod2* expression was significantly increased in these animals ($P < .05$) (Fig. 2). In SOL muscles, there were no differences in mRNA levels of *Atrogin-1*, *Sod2* or *Myh7* between the two sets of treatment groups (Fig. 2). Similarly, pairwise comparisons identified that *Atrogin-1* and *Sod2* expression in TA muscles were not significantly different. There was a decrease in *Myh2* mRNA levels in the SIM80 group compared to the CON80 rats but this was not statistically significant ($P = .0571$) (Fig. 2). In the renal cortices, there were no significant differences in the expression of *GPx*, *Sod1* or *Kim1*. The latter, however, did appear to be reduced by statin treatment but there was large within-group variability (Fig. 3).

3.3. Biochemical analyses

There were no significant differences in serum Ca^{2+}, CK or MG concentrations between CON80 and SIM80 rats but LDH was significantly lower in SIM80 rats (Table 2). There were no significant differences in any of the skeletal muscle biomarkers between the CON50 and SIM50 groups (Table 2). No significant differences in

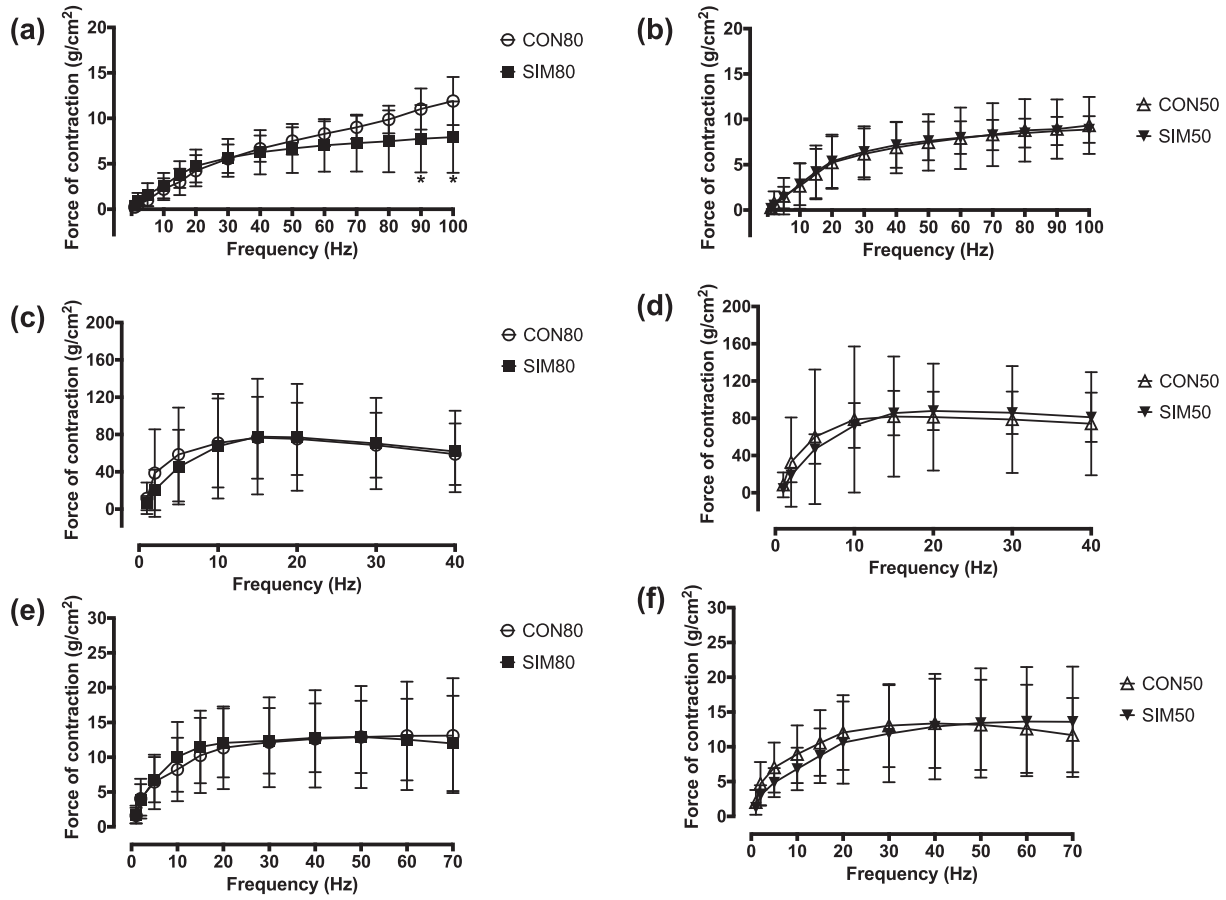

Fig. 1. Normalised force-frequency response curves in isolated muscles from control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats. Data is presented according to pairwise comparisons (CON80 versus SIM80 and CON50 versus SIM50): (a) and (b) show results for isolated gastrocnemius muscles; (c) and (d) show results for isolated soleus muscles and (e) and (f) show results for isolated tibialis anterior muscles. Data are expressed as means with standard deviations (minimum *n* of 9 per group). * Indicates $P < .05$ versus CON80. Significant differences were assessed by two-way repeated measured ANOVA followed by Bonferroni post-hoc test.

Table 1
$Log_{10}EF50$ and maximum force values in isolated gastrocnemius, soleus and tibialis anterior muscles from control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats.

Parameter	CON80	SIM80	CON50	SIM50
Gastrocnemius $Log_{10}EF50$	1.49 (0.18)	1.16 (0.43)*	1.13 (0.33)	1.23 (0.36)
Soleus $Log_{10}EF50$	0.75 (0.79)	0.82 (0.51)	0.86 (0.52)	0.71 (0.30)#
Tibialis anterior $Log_{10}EF50$	0.80 (0.37)	0.83 (0.69)	0.81 (0.42)	0.93 (0.32)#
Gastrocnemius Maximum force generated (g/cm^2)	11.91 (2.65)	8.26 (3.49)*	9.57 (3.25)	9.16 (1.47)
Soleus Maximum force generated (g/cm^2)	93.20 (43.56)	88.45 (59.44)	102.47 (80.74)	95.84 (24.40)
Tibialis anterior Max force generated (g/cm^2)	13.83 (7.98)	15.05 (6.38)	14.13 (6.38)	14.27 (8.15)

Data presented as Mean (SD). Minimum *n* of 9 per group.
* $P < .05$ versus CON80.
# $P < .05$ versus CON50.

creatinine, urea or uric acid were observed between either SIM model and the respective control group (Table 2). There was also no change in the hydroxyproline content of the renal cortices following statin treatment (Table 2).

3.4. Biometric assessments and clinical observations

There were no significant differences in GAS, SOL or TA muscle mass for either pairwise comparison (Table 2). Both statin treatment protocols, however, resulted in a significant increase in kidney mass (appearing as hypertrophy but not fibrosis) compared to the respective control groups ($P < .05$) (Table 2).

All main effects analyses of water intakes and body mass showed significant interaction so simple effects were examined. Water consumption was significantly reduced in both SIM80 and SIM50 animals during the last week of treatment (day 7–14 and day 21–30, respectively) (Fig. 4). In SIM80 rats, significant body mass loss was observed ($P < .05$). Similarly, the SIM50 group exhibited an impaired ability to gain weight from day 7 of treatment ($P < .05$) (Fig. 5). Two SIM80 rats also exhibited decreased activity, a hunched posture and piloerection as well as cold extremities and paralysis of the hind limbs by day 14 of the treatment period. No such characteristics were observed in any of the SIM50 animals.

4. Discussion

This comparative study of two established rodent models of SAMS found that the SIM80 model reproducibly produced changes in skeletal muscle which were characteristic of statin myotoxicity. Contrastingly, the SIM50 treatment protocol did not produce any significant skeletal muscle damage indicating that this model was not as effective in causing symptoms characteristic of SAMS. The findings of this

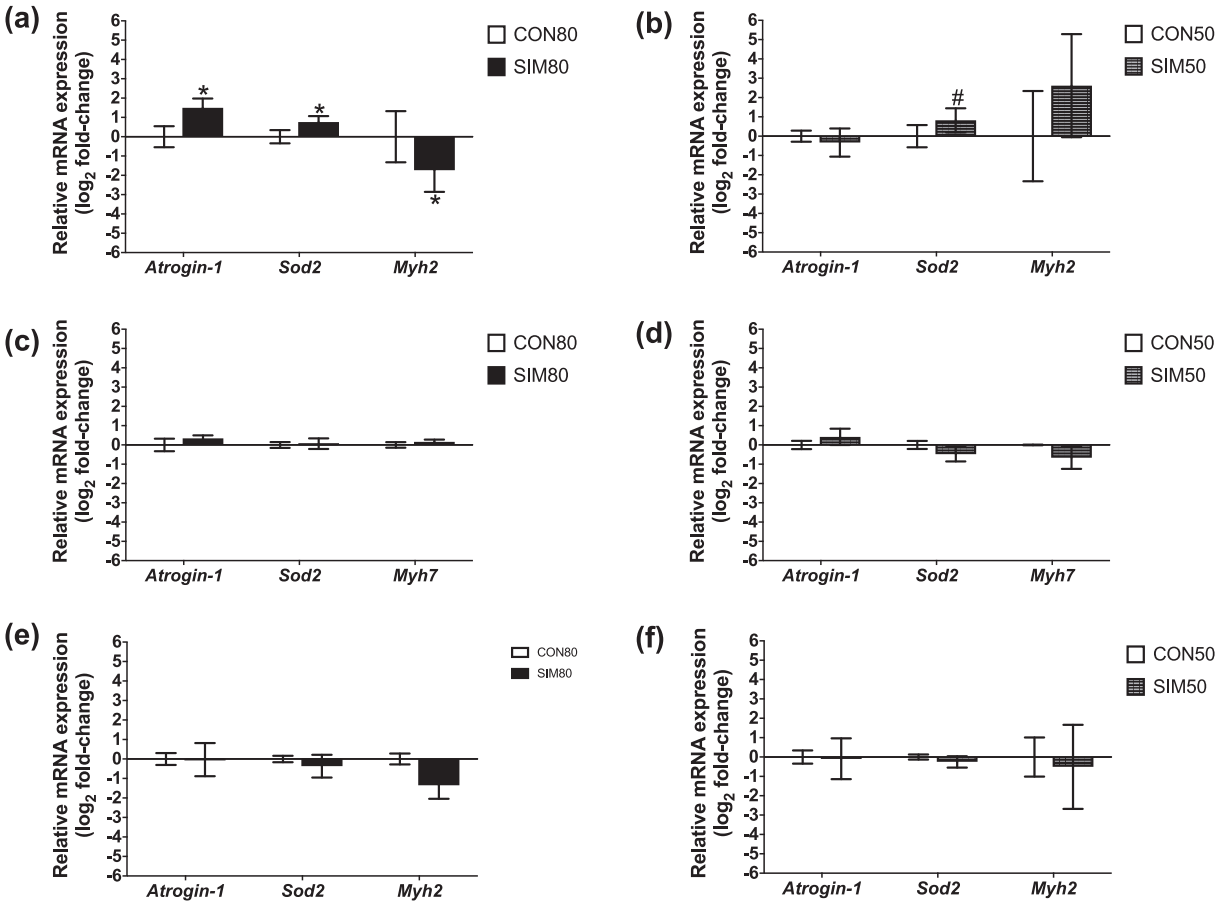

Fig. 2. mRNA expression in isolated muscles from control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats. Data is presented according to pairwise comparisons (CON80 versus SIM80 and CON50 versus SIM50): (a) and (b) show results for isolated gastrocnemius muscles; (c) and (d) show results for isolated soleus muscles and (e) and (f) show results for isolated tibialis anterior muscles. Data are presented as mean Log_2 fold changes means with 95% confidence intervals (minimum *n* of 3 per group). * Indicates $P < .05$ versus CON80 and # indicates $P < .05$ versus CON50. Significant differences were assessed using the Mann-Whitney *U* test.

investigation also validated the use of young, female rats in studies of statin myotoxicity.

Although the clinical manifestation of statin myotoxicity varies considerably between individuals, increased expression of the E3 ubiquitin ligase, *Atrogin-1*, is frequently reported in persons presenting with SAMS (Mallinson et al., 2015; Hanai et al., 2007; Phillips et al., 2010). In the present investigation, neither SAMS model caused gross skeletal muscle atrophy in any of the muscles studied. Nonetheless, GAS muscles isolated from SIM80 rats did show a significant increase in the expression of *Atrogin-1*. Although *Atrogin-1* is linked to skeletal muscle atrophy/proteolysis, increased *Atrogin-1* levels in the absence of atrophy or overt muscle damage has been reported by other rodent studies investigating SAMS (Goodman et al., 2015; Chung et al., 2016). Accordingly, it is postulated that *Atrogin-1* expression precedes the physiological manifestation of SAMS and may thus be one of the critical mediators in statin myotoxicity (Hanai et al., 2007). This concept is consistent with observations in humans whereby the expression of proteolysis-related genes has been noted to be increased in individuals experiencing SAMS while muscle mass has not been reduced (Mallinson et al., 2015).

Although *Atrogin-1* expression itself is not directly correlated with skeletal muscle force production capacity, SIM80 GAS muscles also exhibited a significant reduction in peak force production. Conversely, GAS muscles from SIM50 rats did not show an increase in *Atrogin-1*

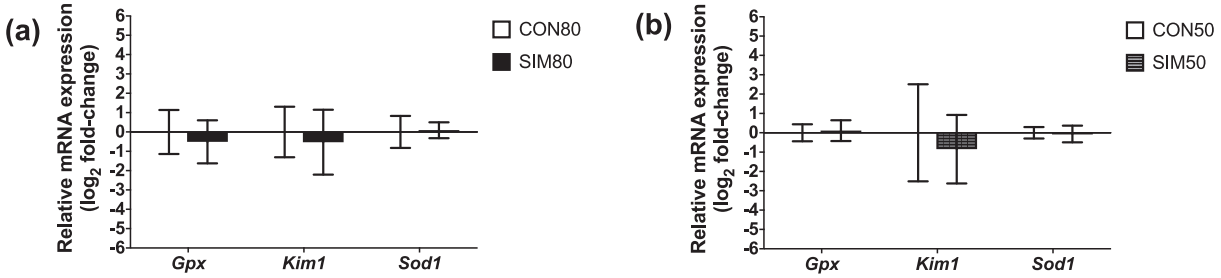

Fig. 3. mRNA expression in isolated renal cortices from control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats. Data is presented according to pairwise comparisons: (a) shows results for CON80 versus SIM80 and (b) shows results for CON50 versus SIM50. Data are presented as mean Log_2 fold changes means with 95% confidence intervals (minimum *n* of 3 per group). Significant differences were assessed using the Mann-Whitney U test.

Table 2
Biometric and physiological parameters in control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats.

Parameter	CON80	SIM80	CON50	SIM50
Gastrocnemius mass (mg gram body mass^{-1})	5.86 (0.50)	5.70 (0.51)	5.66 (0.32)	5.77 (0.52)
Soleus mass (mg gram body mass^{-1})	0.52 (0.07)	0.53 (0.05)	0.49 (0.06)	0.51 (0.07)
Tibialis anterior mass (mg gram body mass^{-1})	1.94 (0.23)	1.86 (0.16)	1.78 (0.15)	1.82 (0.12)
Kidneys mass (mg gram body mass^{-1})	7.27 (0.78)	8.25 (0.93)*	7.10 (0.45)	7.61 (0.80)#
Serum creatine kinase (U L^{-1})	854.80 (596.97)	658.53 (441.60)	838.89 (638.63)	954.5 (493.21)
Serum lactate dehydrogenase (U L^{-1})	1833.57 (493.43)	1208.53 (576.67)*	1706.00 (543.16)	1558.16 (589.93)
Serum myoglobin (μg L^{-1})	19.83 (7.60)	17.52 (5.55)	19.47 (3.59)	23.06 (7.62)
Serum calcium (mmol L^{-1})	2.86 (0.17)	2.88 (0.09)	2.72 (0.18)	2.73 (0.15)
Serum creatinine (μmol L^{-1})	34.11 (4.20)	34.22 (6.59)	34.27 (6.80)	34.33 (3.89)
Serum uric acid (mmol L^{-1})	0.23 (0.09)	0.21 (0.08)	0.22 (0.11)	0.21 (0.07)
Serum urea (mmol L^{-1})	7.36 (1.18)	6.70 (0.63)	6.53 (0.89)	6.19 (1.33)
Renal cortex hydroxyproline content (mg mL^{-1})	5.26 (2.31)	5.18 (2.66)	7.13 (4.67)	6.46 (1.14)

Data presented as Mean (SD). Minimum *n* of 6 per group.
* $P < .05$ versus CON80.
# $P < .05$ versus CON50.

expression, and correspondingly, there was no reduction in peak force relative to CON50 rats. A decrease in force of contraction is frequently observed in fatigued and aging muscle and is consistent with a decline in the force-generating capacity of the muscle (Miljkovic et al., 2015; McGuire et al., 2003). Reduced skeletal muscle force production has been reported in other SAMS rodent studies for multiple statins (Chung et al., 2016; Piette et al., 2016; Meador and Huey, 2011). Furthermore, the effect of STatins On Skeletal Muscle Function and Performance (STOMP) study reported that subjects experiencing myalgia with atorvastatin exhibited decreased muscle strength compared to non-symptomatic statin-treated participants (Parker et al., 2013). Hence, the changes in the GAS muscles isolated from SIM80 rats are consistent with previous reports in both rodents and humans and indicate a decline in the functionality of this fast-twitch muscle.

There were also other changes in the FFCs aside from peak force of contraction which indicated an alteration in the integrity of GAS muscles isolated from SIM80 rats. SIM80 GAS FFCs demonstrated a leftward shift relative to the CON80 group and a subsequent reduction in $Log_{10}EF50$. Previously, this outcome has been correlated to a reduction in the proportion of fast type II fibres to slow-twitch fibres in fast-twitch muscles (Chan and Head, 2010). Consistent with this, rt-qPCR assays identified that *Myh2* mRNA expression was significantly reduced in GAS muscles isolated from SIM80 rats. Similarly, Trapanai et al. observed a 15% shift from faster to slower myosin heavy-chain isoforms and reduced power output in fast-twitch extensor digitorum longus muscles isolated from statin-treated rats (Trapani et al., 2011). Type II muscles fibres (particularly type IIb) are reported to be the most susceptible to statin myotoxicity in both rodents (Westwood et al., 2005; Seachrist et al., 2005) and humans (London et al., 1991). Correspondingly, SOL muscles isolated from both SIM80 and SIM50 rats did not exhibit any significant alterations in functionality or *Atrogin-1* levels. While there were also no significant alterations in force production of the TA muscles, SIM80 rats did show reduced *Myh2* expression in these tissues, albeit not statistically significantly due to inter-group variability in expression levels. Again, this may be indicative of fibre-selective degradation of type II fibres by statin treatment, though not to the same extent as that observed in GAS muscles. It is possible that the higher content of type I fibres in the TA may have provided some resistance against any significant loss of force-generating capacity so a reduction in peak force was not observed. In any case, the clinical implications of changes in the proportion of type I and II muscle fibres in statin-treated individuals remains to be fully elucidated. However, changes in type II fibres are postulated to play an important role in the aging process of human skeletal muscle (Brunner et al., 2007). Hence, statin-induced alterations in type II fibres are likely to have clinically noteworthy implications for muscle function and quality of life in affected individuals.

Contrary to the SIM80 group, evidence of significant skeletal muscle damage was lacking in SIM50 animals. Indeed, in contrast to the SIM80 rats, *Myh2* expression was increased in SIM50 GAS muscles, albeit not significantly again as a consequence of inter-group variability in expression levels. Analysis of FFCs also showed no significant change in GAS $Log_{10}EF50$ in this group relative to CON50 animals. In the SIM50 SOL and TA muscles, however, there was a left- and right-ward shift in FFCs, respectively. The opposite effects have been reported in mice following spinal cord transection (Mrowczynski et al., 2011). Hence, the changes observed in SIM50 SOL and TA values may indicate a resistance to fatigue and improvement in muscle function in these

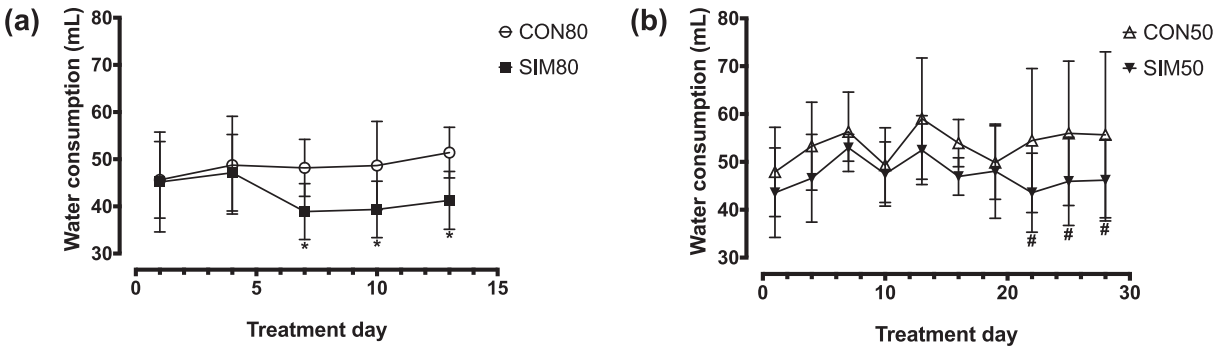

Fig. 4. Water consumption during treatment for control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats. Data is presented according to pairwise comparisons: (a) shows results for CON80 versus SIM80 groups and (b) shows results for CON50 versus SIM50 groups. Data are expressed as means with standard deviations (minimum *n* of 22 per group). * Indicates $P < .05$ versus CON80 curve and # indicates $P < .05$ versus CON50 curve. Significant differences were assessed by two-way repeated measured ANOVA followed by Bonferroni post-hoc test.

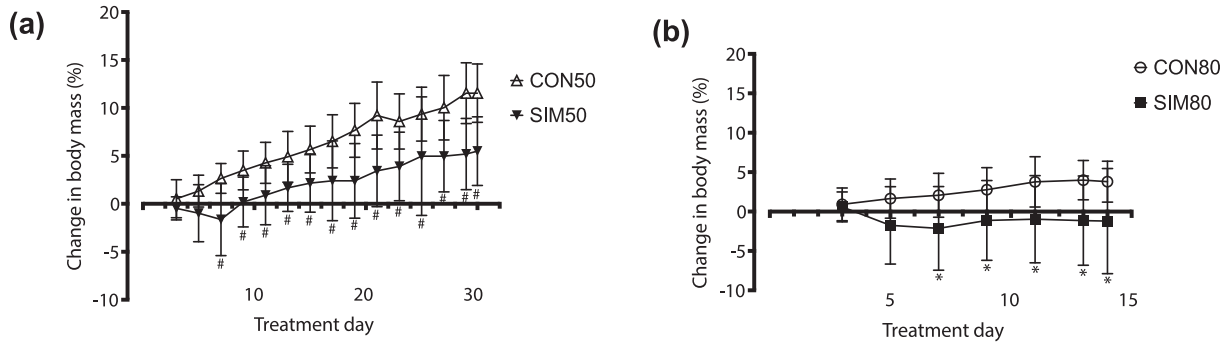

Fig. 5. Percentage change in body mass (normalised to day 1 of treatment) for control (CON80 and CON50) and statin-treated (SIM80 and SIM50) rats. Data is presented according to pairwise comparisons: (a) shows results for CON80 versus SIM80 groups and; (b) shows results for CON50 versus SIM50 groups. Data are expressed as means with standard deviations (minimum *n* of 18 per group). * Indicates $P < .05$ versus CON80 curve and # indicates $P < .05$ versus CON50 curve. Significant differences were assessed by two-way repeated measured ANOVA followed by Bonferroni post-hoc test.

animals. Simvastatin has been reported to have a therapeutic effect on skeletal muscle health in *mdx* dystrophic mice (albeit at very low doses) (Whitehead et al., 2015) indicating that these pharmaceuticals can induce beneficial changes in skeletal muscle integrity. Furthermore, the improvement in the functionality of the SOL observed in this study was not entirely unexpected as unlike type II fibres, type I fibres have been shown in both humans and animals to remain largely unaffected by statin use (Larsen et al., 2013). Nonetheless, the absence of any noteworthy changes in *Atrogin-1* or *Myh2* expression in the GAS or TA muscles indicates that this model did not cause changes in skeletal muscle integrity consistent with previous studies of SAMS.

SOD activity is known to be greatest in muscles with a high proportion of oxidative type I fibres compared to those predominately comprised of type II fibres (Powers et al., 2011). *Sod2* levels were unchanged in SOL and TA muscles from both groups of statin-treated animals, however, expression was significantly increased in the GAS muscles. Increased *Sod2* mRNA levels in the SIM80 GAS muscles may reflect the increased proportion of type I fibres as *Myh2* expression decreased. Nonetheless, as there was no change in *Mhy2* levels in the SIM50 GAS muscles and *Sod2* expression was still increased, this cause seems unlikely. Alternatively, increased oxidative stress in skeletal muscle has been linked to the pathogenesis of statin-induced myotoxicity (Bouitbir et al., 2012; Kwak et al., 2012). Hence, the increase in *Sod2* levels may thus reflect an increase in free radical production in the muscle and subsequent upregulation of *Sod2* to neutralise this effect (Steinbacher and Eckl, 2015). The role of oxidative stress in skeletal muscle is complex so the precise cause of the enhanced *Sod2* expression in GAS muscles remains unclear.

Another factor that may have influenced the differential effect of simvastatin on fast- and slow-twitch muscles is mitochondrial function. While this parameter was not investigated in this study, statin-treated subjects have been shown to exhibit reduced mitochondria volume, decreased levels of mitochondrial DNA as well as reduced expression of oxidative-phosphorylation-related genes in skeletal muscle (Paiva et al., 2005; Hubal et al., 2011). It is postulated that due to their lower mitochondrial content, glycolytic fibres are unable to adapt mitochondrial biogenesis pathways to counteract statin-induced myotoxicity and are thus more susceptible to damage (Bouitbir et al., 2016; Bouitbir et al., 2012). Nonetheless, not all studies have found mitochondrial function to be compromised in subjects experiencing SAMS (Lamperti et al., 2005; Laaksonen et al., 1996). The changes in skeletal muscle physiology observed in this study indicate that the SIM80 model would be suitable for further studies investigating changes in mitochondrial function following statin treatment and thus clarifying its role in the pathogenesis of SAMS.

The absence of muscle atrophy and any significant increase in serum CK, Ca^{2+}, MG or LDH levels in statin-treated rats indicates that neither SAMS model caused significant myonecrosis. The lack of an increase in serum skeletal muscle damage biomarkers in the SIM50 group is consistent with the results from the molecular and organ bath analyses. Although the functional and genetic measures in the SIM80 group indicated a reduction in skeletal muscle integrity, minimal change in plasma CK activity has been reported by other studies using this treatment protocol (Mallinson et al., 2009; Goodman et al., 2015). Moreover, normal plasma CK and/or LDH levels are frequently reported in persons with statin-associated myalgia (Parker and Thompson, 2012; Mallinson et al., 2015). Nonetheless, marked increases in rodent plasma CK with the SIM80 model have been observed (Westwood et al., 2005). The inconsistency in CK measurements between studies does not necessarily indicate a lack of reproducibility of this model as CK levels vary considerably between individuals presenting with SAMS (Rosenson et al., 2014; Muntean et al., 2017). Furthermore, significant variation in CK levels has been seen within individual animal studies (Mallinson et al., 2009), including the present investigation. LDH levels also differed greatly between rats in this study and may have contributed to the higher mean result obtained in the CON80 group when compared to the SIM80 rats. In addition to variability in serum biomarker readings, the absence of significant myonecrosis in the SIM80 rats may indicate that the changes caused by this SAMS model are more characteristic of statin-induced myalgia rather than myonecrosis/rhabdomyolysis. In turn, this validates the clinical relevance of this model as myalgia is the most commonly reported form of SAMS (Thompson et al., 2016). Furthermore, the results from this study also confirm the poor utility of CK and LDH as biomarkers for detecting SAMS in both humans and rodent models of this condition.

Further evidence for the absence of severe rhabdomyolysis is provided by the lack of any significant pathological alteration in kidneys isolated from statin-treated rats. In statin-induced rhabdomyolysis, the presence of excess MG in the renal tubules causes tubular necrosis and acute renal failure (Petejova and Martinek, 2014). In this study, no significant elevation in genes related to oxidative stress or inflammation were observed in kidneys isolated from SIM80 or SIM50 rats. In particular, expression of *Kim1* appeared to be reduced by statin-treated, however, inter-group variability may have omitted any statistically significant effect. Serum biomarkers of renal function were also unchanged in SAMS model rats compared to their respective control groups and there was no evidence of increased collagen (hydroxyproline) deposition in the renal cortex. Despite this, both SIM80 and SIM50 animals exhibited significant renal hypertrophy. In light of the aforementioned biochemical and molecular results, the precise reasons for this increase in kidney mass remains unclear, but renal hypertrophy in the absence of pathology has been reported previously (Williams et al., 2014). In any case, the absence of significant myonecrosis/rhabdomyolysis is not a limitation of the SIM80 model as the different manifestations of SAMS represent a continuum of skeletal muscle damage and thus have the same underlying aetiology (Rosenson et al., 2014;

Parker and Thompson, 2012). Therefore, by causing myalgia and not rhabdomyolysis, the SIM80 model may be ethically and clinically favourable as it can allow for the pathogenesis of statin myotoxicity to be investigated without rodents experiencing a severe terminal pathology.

Nonetheless, some signs of physiological stress, including reduced water intake and an impaired ability to gain weight, were observed in both SIM50 and SIM80 animals. These effects were greater in the SIM80 animals with rats in this group even losing weight during the treatment period. Furthermore, in the SIM50 rats, reduced water consumption and body mass gain were not correlated with myotoxicity. The absence of significant muscle damage in SIM50 rats contrasts considerably with previous observations using this treatment protocol and ultimately indicates a lack of reproducibility of this model (Simsek Ozek et al., 2014). Even so, the lack of any significant myotoxicity with SIM50 treatment was not entirely unexpected. Westwood et al. have reported that for simvastatin-induced myotoxicity to occur in rodents, a minimum dose of 80 mg kg^{-1} day^{-1} is required. Indeed, they observed that lower doses, even when administered for up to 40 days, caused no significant degradation in muscle integrity (Westwood et al., 2005). The findings of the present investigation confirm these observations and also supports that statin dose is likely to be an important mediator in the pathogenesis of SAMS (Buettner and Lecker, 2008; Dujovne et al., 1991).

Although it does produce a clinically-relevant form of SAMS, the use of the SIM80 model is not without its limitations. Specifically, the dose of simvastatin used in this treatment protocol reaches the MTD in rats (Westwood et al., 2005) and as such has the potential to precipitate statin overdose, and potentially, a severe toxicological response. In this study, two SIM80 rats developed cyanosis and paralysis of the hindlimbs by the end of the treatment period. Similar signs of physiological distress (including cold extremities, hunched posture and piloerection) have been reported previously in studies of statin myotoxicity (Westwood et al., 2005; Reijneveld et al., 1996). Nonetheless, these toxicological effects tend to only manifest in a small number of rats and adequate muscle damage can be attained in most of the experimental subjects without these symptoms.

In order to be useful, animal models must produce alterations in physiology that are clinically relevant and representative of the pathology observed in humans. This work has verified that the SIM80 treatment protocol provides a model of statin myotoxicity that is characteristic of that observed in humans. Furthermore, the changes induced by this treatment regimen are clinically relevant as they are akin to the alterations seen in statin-induced myalgia, the most common form of SAMS encountered by clinicians. The SIM80 model will be appropriate for studying the aetiology of SAMS as it provides a baseline against which factors such as age, gender and dose, which are postulated to affect the severity of SAMS, can be assessed (Toth et al., 2018). Likewise, this model provides a suitable platform to compare alternative treatment protocols, such as statin de-challenge and alternate-day dosing (Rosenson et al., 2017), to determine their potential for mitigating statin myotoxicity. The SIM80 treatment regimen can also be used for investigating molecular/metabolic processes which could be targeted to provide novel therapeutic options for the management of SAMS.

In conclusion, this comparative study has shown first-hand that different SAMS models can produce considerably different changes in skeletal muscle integrity. Hence, in order to reduce ambiguity between findings of statin myotoxicity, a standard and reproducible model of SAMS is required. The present study has identified that the SIM80 treatment protocol, when used in young female rodents, is able to reproducibly cause changes in skeletal muscle which are representative of SAMS. These features include fibre-selective increases in *Atrogin-1* expression and oxidative stress, as well as reduced peak force of contraction and/or decreased mRNA expression of *Myh2* in fast-twitch muscles. Hence, it is suggested that the SIM80 model be used for pharmacological studies investigating the pathogenesis of SAMS to elucidate its causes and potential treatment options.

Conflict of interest statement

None to declare.

Financial support

This work was supported by the Australian Government Research Training Scheme. The first author is also supported by the Australian Government Research Training Scheme Stipend.

Declarations of interest

None

Acknowledgements

All major contributors to this work are listed as authors.

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CHAPTER 4

Gene expression profiles in statin-treated rodents with and without myalgia

Preamble

The findings presented in Chapter 3 validated that the SIM80 treatment protocol can be used to elucidate the factors involved in the pathogenesis of statin-induced myalgia. Nevertheless, only a preliminary overview of the genetic changes associated with this particular form of SAMS was completed. Accordingly, the investigation reported in Chapter 4 enhanced and extended these results by assessing a wider range of molecular and biochemical markers. Specifically, Chapter 4 presents the findings of a dose-response study which aimed to compare the molecular, biochemical and functional changes which occur in skeletal muscle following low- and high-dose statin treatment (the latter being the SIM80 model). Ultimately, the findings from this study, in conjunction with the data presented in Chapter 2, served to clarify the involvement of dose in the pathogenesis of SAMS. As studies pertaining to the aetiology of mild forms of SAMS are rare, this investigated presented an opportunity to contribute a significant amount of knowledge to this topic.

This chapter contains a manuscript titled, "Gene expression profiles in statin-treated rodents with and without myalgia". The original version of this work was submitted to *Environmental Toxicology and Pharmacology*; but unfortunately, following peer-review, it was not accepted for publication. A revised version of this manuscript was prepared, submitted to *Muscle and Nerve*, and is currently under review.

Declaration of co-authorship and contribution

Full bibliographic details and Status

Irwin JC, Fenning AS, Vella RK. Gene expression profiles in statin-treated rodents with and without myalgia. Manuscript submitted for publication.
Under review

Nature of Candidate's Contribution, including percentage of total

JC Irwin (85%): Study conceptualisation and design, acquisition of data, analysis and interpretation of data, writing of original draft, critical revision of manuscript and approval of final version.

Nature of all Co-Authors' Contributions, including percentage of total

AS Fenning (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version. RK Vella (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Abstract

Introduction: The genetic changes associated with milder forms of statin-associated muscle symptoms are not fully characterised. This mechanistic dose-response study aimed to address this point using a validated and clinically-relevant rodent model of statin-induced myalgia.

Methods: *Ex vivo* skeletal muscle force production was evaluated, and mRNA levels of genes related to atrophy, mitochondrial biogenesis, metabolism and oxidative stress were assessed. Myosin heavy chain mRNA levels were also measured, as well as tissue hydroxyproline content and serum biomarkers of skeletal muscle damage.

Results: The administration of simvastatin produced a dose-dependent decline in force production in isolated gastrocnemius muscles, but this was not correlated with changes in gene expression or serum biomarkers of muscle damage.

Discussion: The findings of this study suggest that changes in the expression of genes related to atrophy, mitochondrial biogenesis, metabolism and oxidative stress are not closely associated with the development of statin-induced myalgia.

Introduction

The term, statin-associated muscle symptoms (SAMS), describes several distinct adverse muscle-related events which may be experienced with statin therapy [1]. These conditions have unique clinical manifestations and do not present as a continuum of symptoms [2]. Mild variants (such as myalgia) are characterised by muscle pain/fatigue without structural changes in skeletal muscle, while acute forms (e.g. rhabdomyolysis) are exemplified by muscle necrosis and inflammation [3]. These points suggest that the cellular pathogenesis of each form of SAMS varies considerably, but this is yet to be thoroughly investigated.

Rodent-based studies are useful for assessing the molecular alterations associated with statin-induced myotoxicity. Such investigations have implicated atrophy-related pathways [4], mitochondrial dysfunction [5, 6], increased oxidative stress [7] and impaired carbohydrate metabolism [8] in the aetiology of SAMS. The dosing treatments used in these studies, however, have often produced physiological effects which are characteristic of severe myonecrosis/rhabdomyolysis [9, 10]. Thus, considering the disparate clinical presentations of SAMS, it is possible that the genetic changes reported in these studies may only accompany the acute variants of these conditions, as opposed to the milder forms. Hence, further studies are required in order to establish the molecular alterations which accompany milder forms of SAMS. Identifying these changes is integral for elucidating the pathogenesis of statin-induced muscle pain/fatigue, and thus potential interventions which can be used to manage these symptoms.

We have recently validated a dosing protocol which induces physiological changes in rodents that are clinically-relevant and characteristic of satin-induced myalgia observed in humans (i.e.

fiber-selective muscle fatigue in the absence of elevated serum creatine kinase (CK)) [11]. The present investigation aimed to use this model in order to evaluate the genetic changes which accompany statin-induced muscle fatigue. This study employed a dose-response design; thus, the mRNA expression profiles of statin-treated rats with myalgia could be compared to those from asymptomatic statin-treated animals. In turn, this enabled the associations (or lack thereof) between changes in gene expressions and skeletal muscle performance to be more rigorously assessed.

Methods

Ethical approval

This study was approved by the Animal Ethics Committee of Central Queensland University (AEC: 20217) following guidelines from the National Medical Research Council of Australia (NHMRC). Rodents were provided water and food *ad libitum* and were housed at 22 ± 2^{o}C on a 12-hour light/12-hour dark cycle. To monitor the health of the animals during the treatment period, body mass and water intake were measured every 2 and 3 days, respectively. At the completion of the treatment period, rodents were euthanised via a 1.0 mL intraperitoneal injection of sodium pentobarbitone (187.5 mg mL^{-1}), and death was confirmed by the absence of pedal and corneal reflexes.

Animals and pharmacological treatments

Female Wistar rats (Central Queensland University Rodent Breeding Colony, Rockhampton) were randomised to one of three treatment groups: statin-induced myalgia model (80 mg kg^{-1}

of simvastatin) (SIM80, $n = 25$), low-dose statin treatment (40 mg kg^{-1} of simvastatin) (SIM40, $n = 25$) or control (no intervention given) (CON, $n = 25$). All interventions were administered for 14 days via oral gavage. Simvastatin tablets (Rockhampton Base Hospital, Australia) were crushed using a mortar and pestle and dissolved in a 10% v/v solution of Polysorbate20 in milli-Q water for delivery [11].

Muscle mass

Following euthanasia, the gastrocnemius, soleus and tibialis anterior muscles were isolated and their wet mass (as normalised to body mass) was recorded. These muscles were selected as they are of variable myosin heavy chain (MHC) fiber type composition. Estimated populations (%) of MHC type I, MHC type IIA, MHC type IIB and MHC type IID/X fibers (respectively) are as follows: gastrocnemius – 3, 6, 57 and 34; soleus – 84, 7, <1 and 9; tibialis anterior – 2, 18, 46 and 34 [12].

Ex vivo assessment of skeletal muscle performance

Electrical field stimulation was performed to produce force-frequency curves (FFCs), as per an established protocol [11]. In brief, the isolated muscles were exposed to stimulation trains of 5 seconds in duration (pulses 0.1 ms at 100 V) at increasing intervals of 1 to 100 Hz (135 seconds rest between stimulations). Force responses were detected using Grass FT03 transducers and recorded using Lab Chart software and PowerLab® data acquisition units (ADInstruments, Bella Vista, Australia). Non-linear regression of FFCs was used to calculate $Log_{10}EF50$ values. Data up to 100 Hz, 40 Hz and 70 Hz for gastrocnemius, soleus and tibialis

anterior muscles, respectively, were used for the non-linear regression analyses to ensure that accurate calculations of $Log_{10}EF50$ values were obtained.

Quantitative reverse transcription PCR (RT-qPCR)

The mRNA for the RT-qPCR assays was extracted from randomly selected samples (n = 6) using Trizol™ reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of mRNA was evaluated using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific), and samples were reverse transcribed to cDNA using Superscript™ III reverse transcriptase (Applied Biosystems Inc.). PCR reactions were run using Taqman™ universal PCR master mix (Applied Biosystems Inc.) and Rotor-Gene® Q equipment (Qiagen). The full list of genes investigated is provided in **Table 1**. Samples with *Ct* values > 35 were excluded and No-RT controls were acceptable if *Ct* values were $\geq$ 5 cycles compared to samples [13]. Relative expression (normalised to *Gapdh* (Rn01775763_g1)) was calculated [14] and statistical analysis was performed on the delta *Ct* values.

Serum biomarkers

Serum creatine kinase MM fraction (CK-MM), myoglobin and heart-type fatty acid binding protein (H-FABP) were measured in randomly selected samples (n = 10) using commercially available ELISA kits (Fisher Biotec Australia). The latter two biomarkers were used to calculate the myoglobin/H-FABP ratio (a ratio between 21 and 73 was indicative of skeletal muscle injury [15]). The collagen content in randomly selected skeletal muscle samples (n = 11) was measured using a modified colourimetric hydroxyproline assay [11].

Data and statistical analysis

Prior to statistical analysis, datasets were examined for outliers using the ROUT analysis method. Following this testing, normality was assessed using the D'Agostino-Pearson test or Kolomogov-Smirnov test (depending on sample size). Parametric data was analysed using one-way ANOVA, or two-way ANOVA, with Tukey post-hoc tests when required (alpha < 0.05). Data that was not normally distributed was assessed using the Kruskal-Wallis test with Dunn's post-hoc testing. All analyses were conducted using GraphPad Prism 8 (GraphPad Software, La Jolla, USA) and data are presented as means (SD).

Results

Simvastatin caused a dose-dependent reduction in force production in gastrocnemius muscles

Simple effects two-way ANOVA identified that the force production of gastrocnemius muscles isolated from the SIM80 rats was significantly lower than the other treatment groups. Specifically, the CON and SIM40 groups exhibited greater performance compared to the SIM80 animals from 80 Hz ($P < 0.01$) and 60 Hz onwards ($P < 0.05$), respectively (**Fig. 1**). Maximum force production was reduced in the SIM80 rats compared to the CON animals ($P = 0.02$), however high within-group variability negated any statistically significant effect in comparison to the SIM40 group ($P = 0.09$) (**Table 2**). Non-linear regression analysis identified a significant reduction in $Log_{10}EF50$ for the SIM80 rats compared to the CON and SIM40 groups ($P < 0.0001$) (**Table 2**). There were no differences in the performance of gastrocnemius muscles between the CON and SIM40 groups.

Force production appeared to be increased in the tibialis anterior muscles of the SIM40 animals, however, there were no significant differences in maximum force production amongst the treatment groups. Furthermore, simple effects analysis only identified a statistically significant difference in performance between the CON and SIM40 animals at 40 Hz (P = 0.04). The SIM40 rats had a higher $Log_{10}EF50$ value in comparison to the other treatment groups, and this was statistically significant relative to the CON group (P = 0.0004) (**Table 2**). There were no significant differences in FFCs, maximum force production or $Log_{10}EF50$ in the soleus muscles.

Simvastatin did not cause muscle atrophy, but did reduce water consumption

Statin treatment was not associated with any loss of muscle mass (**Table 3**). Simple effects two-way ANOVA identified that water intake was significantly reduced in both the SIM80 and SIM40 animals for the whole of the treatment period ($P < 0.05$ versus CON) (**Fig. 2**). Water intake was also significantly lower in the SIM40 animals compared to the SIM80 group on days 4 and 10 of treatment ($P < 0.0001$). This effect was not accompanied by other signs of physiological stress (e.g. loss of body mass) and was concluded not to be pathological. The SIM80 rats exhibited reduced body weight gain during the first week of the dosing, but this effect was not statistically significant and had normalised by the end of the treatment period.

Loss of skeletal muscle performance in statin-induced myalgia occurs without changes in genes related to atrophy, mitochondrial dysfunction, metabolism or oxidative stress

In isolated gastrocnemius muscles, no significant differences in the mRNA levels of *Atrogin-1*, *Murf-1*, *Mstn* or *Ctsl* were observed in the SIM80 or SIM40 rats (**Fig. 3**). The mRNA levels

of MHC isoforms also showed no significant differences, though there were 3-to-8-fold reductions in the expression of MHC type I and MHC type IIA fibers in the statin-treated groups. Nevertheless, there was very high within-group variability in mRNA levels and this indicated a lack of a strong treatment-induced effect. There were little-to-no changes in the expression of *Pgc-1α*, *Pdk4*, *Pparα*, *Ucp3*, *Mt1a* or *Sod2* in isolated gastrocnemius muscles. *Nox2* mRNA levels were 5-fold higher in SIM80 animals compared to SIM40 rats ($P = 0.02$); however, neither group exhibited statistically significant differences in expression in comparison to the CON animals.

There were no changes in the expression of genes related to atrophy or protein degradation in the soleus muscles of the statin-treated animals (**Fig. 3**). Similarly, no changes in the expression of MHC type I fibers were observed. There were mild differences in the mRNA levels of MHC type IIA and MHC type IID/X fibers; however, owing to within-group heterogeneity, none were statistically significant. The mRNA levels of MHC type IIB fibers were significantly lower in the soleus muscles isolated from the SIM80 animals compared to the CON group ($P < 0.02$). The mRNA levels of *Pgc-1α*, *Pdk4*, *Pparα* and *Ucp3* were not significantly affected by statin-treatment, though a small 3-fold increase in *Pdk4* mRNA levels was observed in the SIM40 group. There were no significant differences in *Sod2* or *Nox2* expression amongst the treatment groups. *Mt1a* mRNA expression was lower in the SIM80 rats compared to the SIM40 animals ($P = 0.02$) but was not significantly different compared to the CON group.

Statin administration caused no significant changes in the expression of atrophy-related genes in isolated tibialis anterior muscles, and there were no differences in the mRNA levels of MHCs. *Ctsl* expression was slightly increased (approximately 3-fold) in the SIM80 rats, but this was

not a statistically significant effect. *Pgc-1α* mRNA levels were unaltered by statin administration. Statin-treated rats exhibited 3-to-4-fold changes in *Pdk4* expression, and this effect was statistically significant in the SIM40 group relative to the CON animals ($P = 0.01$). There was a mild increase in *Ucp3* expression in the SIM40 animals compared to the other treatment groups, however this change was not statistically significant. The mRNA levels of *Pparα* and *Nox2* were significantly lower in the SIM40 animals relative to the SIM80 rats ($P = 0.04$ and 0.02, respectively). The expressions of these genes were also lower in comparison to the CON group, but these changes were not statistically significant. While there was a 10-fold difference in *Nox2* expression the between SIM80 and SIM40 animals, the actual difference in *Pparα* expression was minimal. There was little change in *Sod2* expression following statin administration. *Mt1a* expression was largely unchanged by SIM40 treatment but was increased 9-fold in the SIM80 group relative to the CON rats. However, there was high within-group variability in the expression of this gene, thereby indicating the lack of a strong-treatment-induced effect.

Serum biomarkers of skeletal muscle damage did not correlate with muscle performance

Average serum concentrations of H-FABP and CK-MM were two and three times greater, respectively, in the SIM80 group compared to the CON animals. Nonetheless, there were high individual values in the SIM80 group which had increased the group average for these biomarkers. These values presumably had not been identified as outliers by the ROUT analysis owing to the large within-group variability. Accordingly, no statistically significant differences between the SIM80 and CON rats were identified for these biomarkers (**Fig. 4**). Similarly, there were no significant differences in serum concentrations of myoglobin between the SIM80 and CON animals. Conversely, SIM40 rats exhibited significantly higher serum levels of all

biomarkers in comparison to both the CON and SIM80 groups ($P < 0.05$) (**Fig. 4**). Again, however, values were largely heterogeneous, particularly for H-FABP and CK-MM. The average myoglobin/H-FABP ratio was similar between the CON and SIM80 groups but lower in the SIM40 group. Nevertheless, there was high within-group variability meaning no statistically significant differences were observed.

There was also large heterogeneity in the hydroxyproline content of the skeletal muscles (**Fig. 5**). In both statin-treated groups, high individual values increased the average concentration of hydroxyproline. Ultimately, the lack of any homogeneity in the group values indicated a lack of a strong treatment-induced effect and thus no statistically significant differences were observed.

Discussion

Statin treatment caused a dose-dependent decline in the performance of isolated gastrocnemius muscles. Skeletal muscle fatigue in the SIM80 rats was evidenced by a reduction in contractile force, as well as a leftward shift in the FFC [16]. Owing to its high proportion of fast-twitch glycolytic fibers, the loss of force observed within the SIM80 rats was expected and is consistent with our previous findings using this model [11]. Moreover, muscles containing predominately glycolytic fibers (i.e. MHC type IIB and MHC type IID/X fibers) have been repeatedly observed to be the most adversely affected by statin administration [9, 11, 17-19]. The absence of muscle fatigue in the SIM40 rats corroborated previous reports that the myotoxic effects of statins are correlated with dose [9, 11, 20-22].

The loss of contractile force in the SIM80 gastrocnemius muscles occurred without overt structural changes in muscle integrity, as evidenced by the absence of muscle atrophy or altered hydroxyproline levels. The mRNA levels of *Atrogin-1*, *Murf-1*, *Mstn* and *Ctsl* were also unchanged in the presence of statin-induced muscle fatigue. These findings are in direct contrast to reports of elevated *Atrogin-1* [4, 23], *Murf-1* [24, 25] and collagen levels [7] in other rodent-based studies of SAMS. Indeed, increased *Atrogin-1* expression is postulated to be a key factor in the pathogenesis of SAMS [4, 26]. Nevertheless, other studies have shown that *Atrogin-1* expression is not correlated with skeletal muscle performance [27, 28]. Similarly, the results of the present investigation indicate that statin-induced muscle fatigue is not always accompanied by changes in the expression of genes related to muscle atrophy or protein degradation.

Alterations in MHC fiber type expression were also not associated with force production. For instance, SIM40 and SIM80 gastrocnemius muscles exhibited similar MHC mRNA levels, but the functional performance of these tissues differed significantly. Similarly, while the SIM80 soleus muscles exhibited reduced mRNA levels of MHC type IIB, this was not accompanied by any change in muscle function. The mRNA levels of the MHCs also exhibited considerable within-group variability which suggests that the observed differences in gene expression may not have been solely attributable to statin treatment.

The onset of SAMS has previously been associated with reduced mitochondrial biogenesis and/or mitochondrial dysfunction [5, 18, 29, 30]. In the present study, however, no correlation between *Pgc-1α* or *Ucp3* expression and skeletal muscle performance was observed. Although mitochondrial dysfunction is often linked with the onset of statin-induced myotoxicity, others have reported no change in mitochondrial content/activity in subjects experiencing SAMS [31,

32]. Therefore, these findings suggest that the development of SAMS may not always be intrinsically linked with altered mitochondrial integrity.

Reduced force production in the SIM80 gastrocnemius muscles was not accompanied by an increase in the expression of oxidative stress-related genes relative to the CON group. This finding contrasts with other reports of significant increases in the expression of ROS-generating and/or antioxidant genes during SAMS [33, 34]. Indeed, we have previously reported an increase in *Sod2* expression in gastrocnemius muscles following SIM80 treatment [11]. While the exact cause for the discrepancy is unclear, this inconsistency suggests that statin-induced muscle fatigue may not be as strongly correlated with *Sod2* expression as previously suggested. *Mt1a* mRNA levels were variable (particularly in the tibialis anterior) and similarly did not correlate with skeletal muscle performance. For instance, while *Mt1a* expression was significantly reduced in soleus muscles isolated from the SIM80 rats compared to the SIM40 animals, both groups exhibited comparable force production.

The only gene to demonstrate a dose-dependent change in expression following statin treatment which reflected the functional performance of the muscles was *Nox2*. Specifically, both the gastrocnemius and tibialis anterior muscles isolated from the SIM40 rats exhibited significantly reduced mRNA levels of *Nox2* compared to the SIM80 animals. Correspondingly, the SIM40 gastrocnemius and tibialis anterior muscles performed better than those from the SIM80 group. Nonetheless, the relationship between *Nox2* expression and force production was not consistent across the entire dataset. For example, CON and SIM80 gastrocnemius muscles showed comparable mRNA levels of *Nox2*, but the functional performance of these tissues differed significantly. To the best of our knowledge, this is amongst the first investigations to measure

Nox2 expression in a rodent model of SAMS. Further studies to clarify the association between *Nox2* and statin-induced muscle fatigue are thus required.

The onset of SAMS has been linked to impaired muscle metabolism (particularly reduced carbohydrate catabolism) in previous studies [8, 33, 35]. In the present investigation, however, there was no clear correlation between *Pdk4* or *Pparα* mRNA levels and the development of statin-induced muscle fatigue. For instance, the SIM40 and SIM80 tibialis anterior muscles exhibited a comparable increase in *Pdk4* expression, however, muscle performance was greater in the SIM40 rats. Likewise, despite exhibiting muscle fatigue, there was no increase in *Pdk4* in the SIM80 gastrocnemius muscles. This result contrasts the work of Mallinson et al. which demonstrated a near 15-fold increase in *Pdk4* mRNA levels in predominately fast-twitch glycolytic muscles (i.e. biceps femoris) following four days of SIM80 treatment [8]. The rodents employed by Mallinson et al. exhibited a more acute form of SAMS (i.e. muscle necrosis with serum CK elevation) compared to the SIM80 rats in the present investigation. Hence, increased *Pdk4* expression may only be associated with more severe variants of SAMS. This point is supported by another study from Mallinson and colleagues which found protein levels of *Pdk4* to be unchanged between non-statin users and individuals with statin-induced myalgia [36].

Akin to the molecular alterations, changes in serum biomarkers of skeletal muscle damage did not exhibit a dose-response effect, and they were not consistent with the functional and biometric evaluations. For instance, the CON group exhibited an average myoglobin/H-FABP ratio > 21 (indicating significant muscle injury), but there was no evidence of myotoxicity in the functional and biometric analyses. Similarly, the SIM40 group exhibited the highest serum concentrations of myoglobin, H-FABP and CK-MM, but muscle fatigue/atrophy was absent in

this group. The concentrations of these biomarkers also exhibited considerable within-group variability. Serum muscle protein values have previously been reported to be unreliable for monitoring myopathic events, including statin-induced myotoxicity [37, 38]. The findings of this present study corroborate these observations and suggest that the usefulness of these biomarkers for monitoring SAMS is limited.

Ultimately, this investigation has demonstrated that the expression of genes related to muscle atrophy, mitochondrial function, oxidative stress and metabolism are not strongly correlated with the onset of statin-induced myalgia. The disparity between the gene expression profiles reported in this work and previous rodent-based studies of SAMS indicates that the molecular alterations which occur in mild and severe variants of SAMS differ significantly. Thus, the results of rodent-based studies of SAMS should not be generalised to encompass all forms of statin-induced myotoxicity. Alternatively, future investigations of SAMS should employ rodent models that are representative of a specific clinical manifestation of SAMS (such as the model of statin-induced myalgia used in this work) in order to more accurately elucidate the pathogenesis of these conditions.

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Figure Legend

Fig. 1 Normalised force-frequency response curves in muscles isolated from control (CON) and statin-treated (SIM80 and SIM40) rats: (A) shows results for gastrocnemius muscles; (B) for soleus muscles and; (C) for tibialis anterior muscles. Results are expressed as means with standard deviations (*n* of 13-23 per group following removal of inviable tissues and outliers by ROUT analysis). This data was analysed using two-way repeated measures ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and # indicates $P < 0.05$ versus SIM80.

Fig. 2 (A) Water consumption and (B) percentage change in body mass during treatment for control (CON) and statin-treated (SIM80 and SIM40) rats. Percentage change in body mass is normalised to day 1 of treatment. Results are expressed as means with standard deviations (*n* of 19-24 per group following removal of outliers by ROUT analysis). This data was analysed using two-way repeated measures ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and # indicates $P < 0.05$ versus SIM80.

Fig. 3 mRNA expression in skeletal muscles isolated from control (CON) and statin-treated (SIM80 and SIM40) rats: (A) shows results for gastrocnemius muscles; (B) for soleus muscles and; (C) for tibialis anterior muscles. Results are expressed as mean Log_2 fold changes with standard deviations (*n* of 5-6 per group following removal of outliers by ROUT analysis). This data was analysed using one-way ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and # indicates $P < 0.05$ versus SIM80.

Fig. 4 Scatterplots of individual levels of serum biomarkers from control (CON) and statin-treated (SIM80 and SIM40) rats: (A) shows results for myoglobin; (B) for heart-type fatty acid

binding protein (H-FABP); (C) for creatine kinase MM fraction (CK-MM) and; (D) for the myoglobin/H-FABP ratio. Means are depicted by horizontal lines (*n* of 7-10 per group following removal of outliers by ROUT analysis). This data was analysed using one-way ANOVA followed by Tukey post-hoc test, aside from myoglobin which was assessed using the Kruskal-Wallis test. * indicates $P < 0.05$ versus CON and $^{\#}$ indicates $P < 0.05$ versus SIM80.

Fig. 5 Scatterplots of individual levels of hydroxyproline content from control (CON) and statin-treated (SIM80 and SIM40) rats: (A) shows results for gastrocnemius muscles; (B) for soleus muscles and; (C) for tibialis anterior muscles. Means are depicted by horizontal lines (*n* of 9-11 per group following removal of outliers by ROUT analysis). This data was analysed using one-way ANOVA followed by Tukey post-hoc test.

Table 1 Gene expression assays

Gene of interest	Taqman™ Expression Assay	Amplicon length
Mitochondrial biogenesis/dysfunction		
Ppargc1α (*Pgc-1α*)	Rn00580241_m1	94
Ucp3	Rn00565874_m1	80
Atrophy and protein degradation		
Fbxo32 (*Atrogin-1*)	Rn00591730_m1	61
Murf-1 (*Trim63*)	Rn00590197_m1	56
Mstn	Rn00569683_m1	67
Ctsl	Rn04341361_m1	92
Myosin heavy chain isoform		
Myh7 (MHC type I)	Rn01488777_g1	76
Myh2 (MHC type IIA)	Rn01470656_m1	82
Myh4 (MHC type IIB)	Rn01496087_g1	65
Myh1 (MHC type D/X)	Rn01751056_m1	71
Metabolism		
Pdk4	Rn00585577_m1	76
Pparα	Rn00566193_m1	98
Oxidative stress		
Cybb (*Nox2*)	Rn00576710_m1	77
Sod2	Rn00690588_g1	64
Mt1a	Rn00821759_g1	88

Table 2 $Log_{10}EF50$ and maximum force values of gastrocnemius, soleus and tibialis anterior muscles isolated from control (CON) and statin-treated (SIM40 and SIM80) rats

Parameter	CON	SIM40	SIM80
Gastrocnemius $Log_{10}EF50$	1.46(0.22)	1.38(0.305)$^{\#}$	1.23(0.45)*
Soleus $Log_{10}EF50$	0.75(0.59)	0.76(0.48)	0.83(0.56)
Tibialis anterior $Log_{10}EF50$	0.87(0.41)	1.01(0.32)*	0.92(0.44)
Gastrocnemius Maximum force (g/cm^2)	12.19(3.51)	11.04(3.85)	8.56(3.38)*
Soleus Maximum force (g/cm^2)	123.10(70.11)	125.40(64.21)	100.60(66.65)
Tibialis anterior Maximum force (g/cm^2)	16.38(8.91)	20.04(8.31)	16.44(4.63)

Results are expressed as means with standard deviations. *n* of 13-23 per group following removal of inviable tissues and outliers by ROUT analysis. Data was analysed using one-way ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and $^{\#}$ indicates $P < 0.05$ versus SIM80.

Table 3 Mass of gastrocnemius, soleus and tibialis anterior muscles isolated from control (CON) and statin-treated (SIM40 and SIM80) rats

Parameter	CON	SIM40	SIM80
Gastrocnemius mass (mg gram body mass^{-1})	5.87(0.49)	5.86(0.46)	5.66(0.50)
Soleus mass (mg gram body mass^{-1})	0.53(0.09)	0.55(0.08)	0.53(0.08)
Tibialis anterior mass (mg gram body mass^{-1})	1.99(0.28)	1.92(0.21)	1.88(0.25)

Results are expressed as means with standard deviations. *n* of 23-24 per group for maximum force following removal of outliers by ROUT analysis. Data was analysed using one-way ANOVA followed by Tukey post-hoc test.

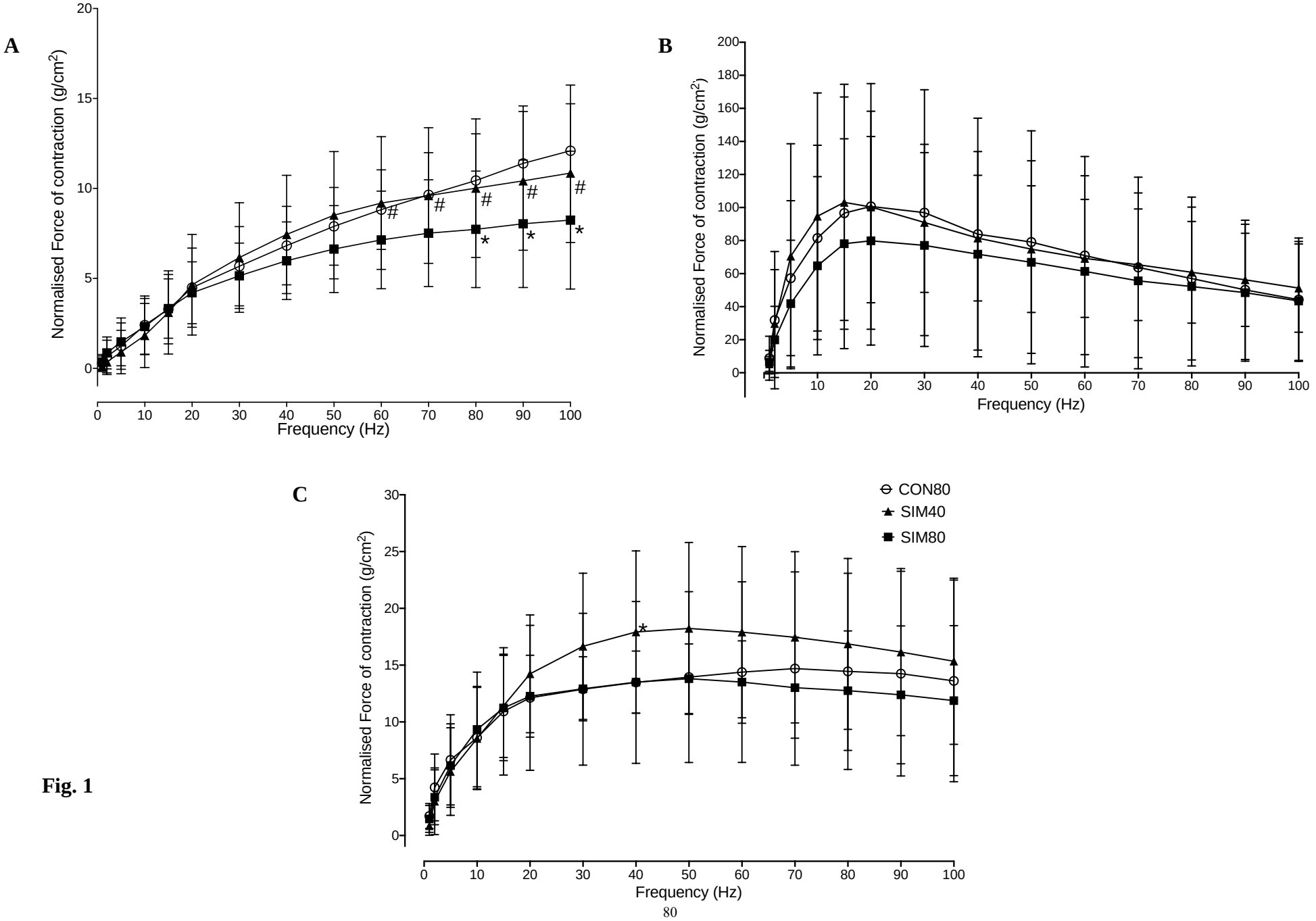
A
Normalised Force of contraction (g/cm2)
Frequency (Hz)
B
Normalised Force of contraction (g/cm2)
Frequency (Hz)
C
CON80
SIM40
SIM80
Normalised Force of contraction (g/cm2)
Frequency (Hz)

Fig. 1

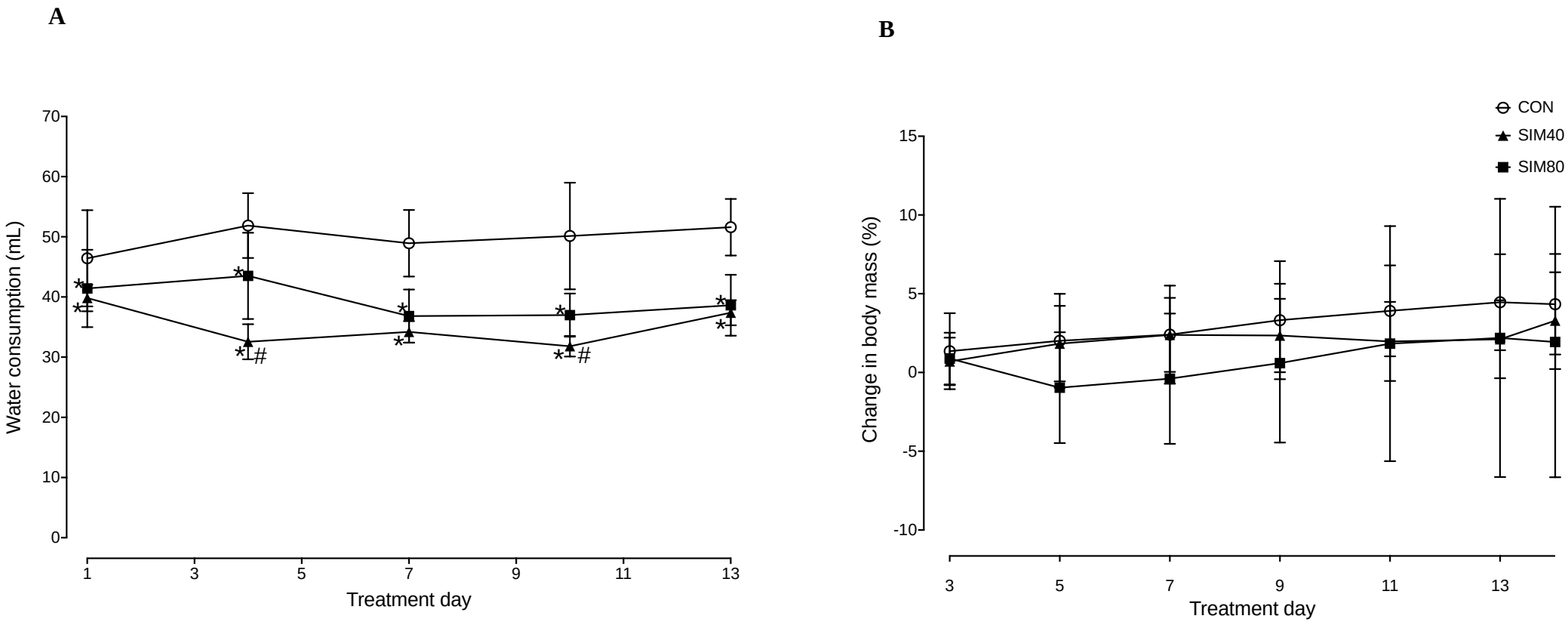
A
B
Water consumption (mL)
Treatment day
Change in body mass (%)
CON
SIM40
SIM80

Fig. 2

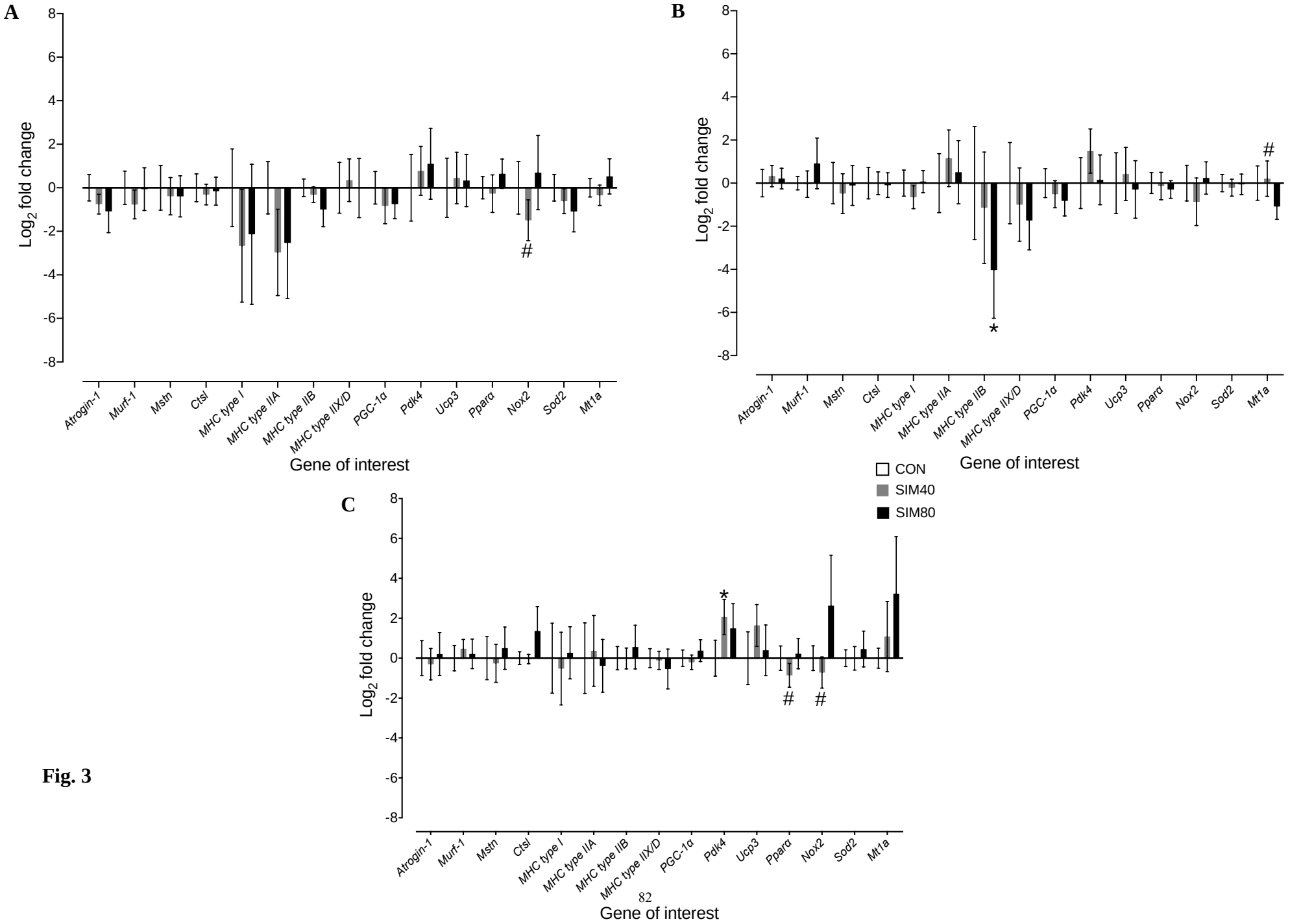
A
B
C
Log2 fold change
Gene of interest
Atrogin-1
Murf-1
Mstn
Ctsl
MHC type I
MHC type IIA
MHC type IIB
MHC type IIX/D
PGC-1α
Pdk4
Ucp3
Pparα
Nox2
Sod2
Mt1a
CON
SIM40
SIM80
#
*

Fig. 3

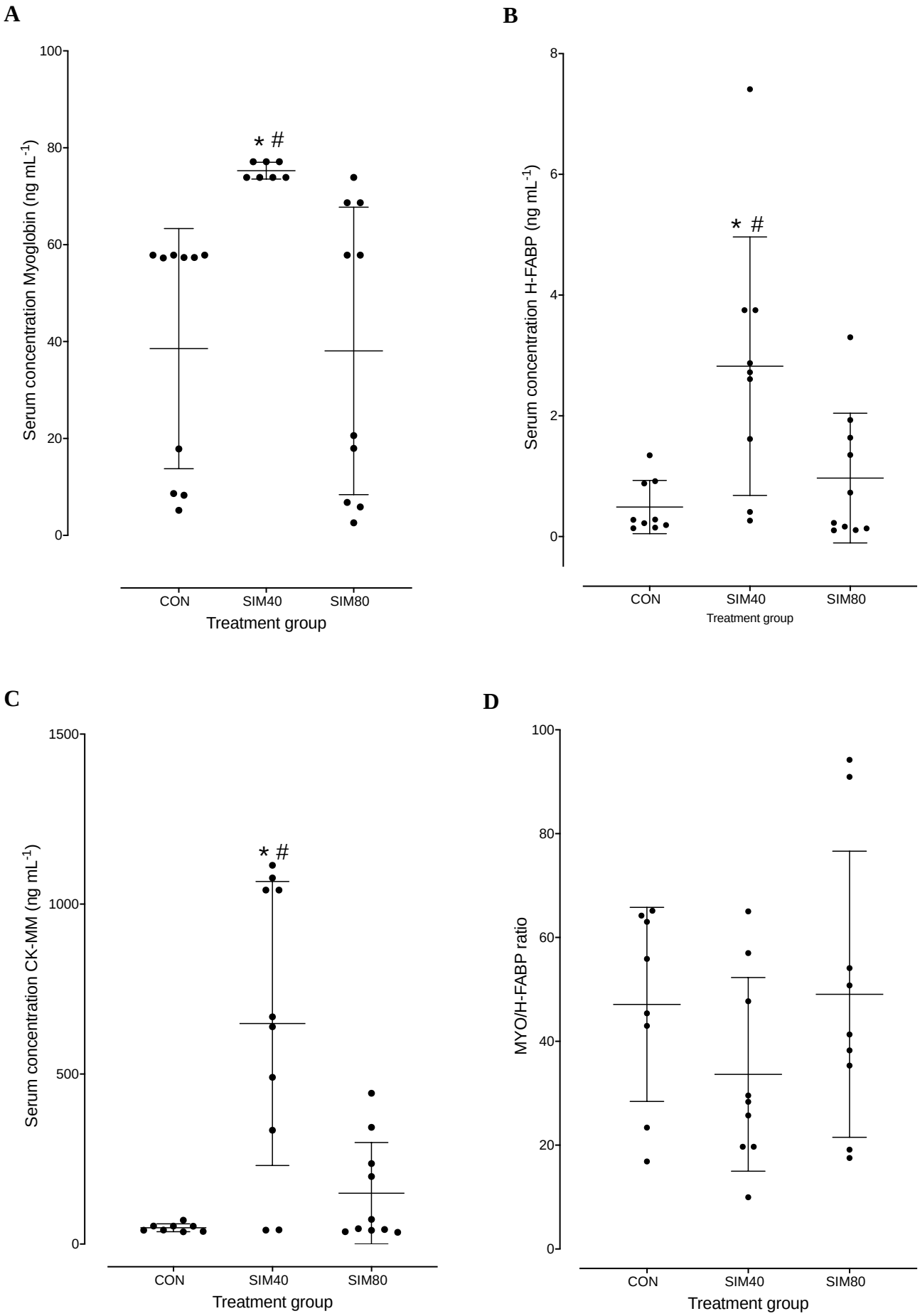
A
Serum concentration Myoglobin (ng mL-1)
100
80
60
40
20
0
* #
CON
SIM40
SIM80
Treatment group
B
Serum concentration H-FABP (ng mL-1)
8
6
4
2
0
* #
CON
SIM40
SIM80
Treatment group
C
Serum concentration CK-MM (ng mL-1)
1500
1000
500
0
* #
CON
SIM40
SIM80
Treatment group
D
MYO/H-FABP ratio
100
80
60
40
20
0
CON
SIM40
SIM80
Treatment group

Fig. 4

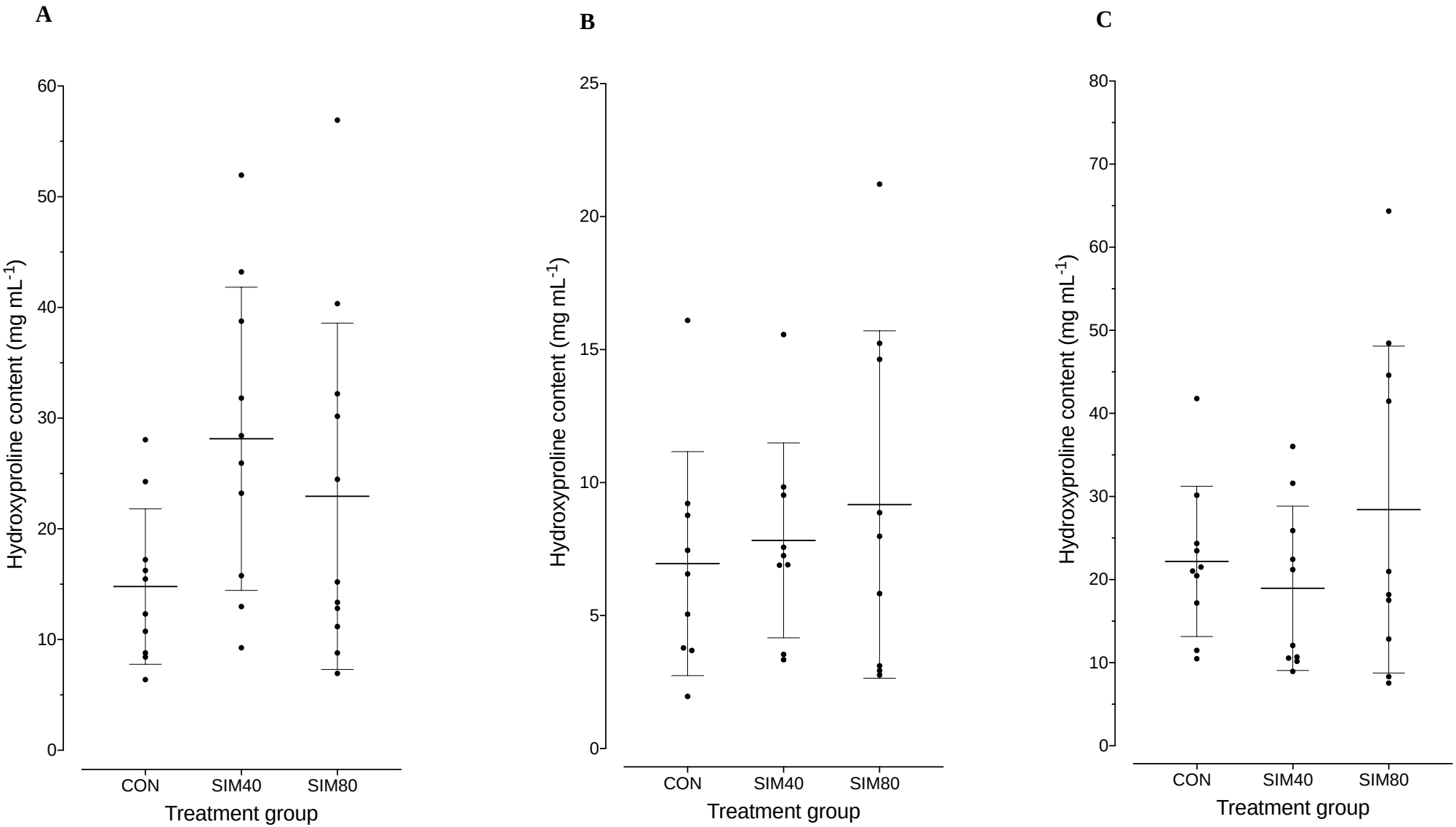
A
B
C
Hydroxyproline content (mg mL-1)
Treatment group
CON
SIM40
SIM80
0
10
20
30
40
50
60
5
15
25
70
80

Fig. 5

CHAPTER 5

Statins with different lipophilic indices exert distinct effects on skeletal, cardiac and vascular smooth muscle

Preamble

The findings presented in Chapter 4 clarified that statin dose can significantly influence the potential of these pharmaceuticals to cause myalgia. Accordingly, the next investigation in this project aimed to determine whether the effects of statins on skeletal muscle function also varied according to their lipophilic index. Additionally, this study served to extend the findings of the previous chapters by evaluating whether cardiac and/or vascular smooth muscle physiology was altered in the presence of statin-induced myalgia.

Biochemical markers of skeletal muscle damage were not measured in this investigation owing to their unreliability in monitoring the progression of statin-induced myalgia in rodents (as detailed in Chapter 4). However, mRNA levels of genes related to muscle atrophy, impaired metabolism and oxidative stress were still assessed, even though the previous study found no correlation between their expression and skeletal muscle performance. It was necessary to complete these analyses as the SIM80 gene expression profiles differed between the studies reported in Chapters 3 and 4. As these inconsistencies challenged the reproducibility of the SIM80 model, it was pertinent to establish whether this variation continued (see section 7.8.2. for further discussion).

This chapter contains a manuscript titled, “Statins with different lipophilic indices exert distinct effects on skeletal, cardiac and vascular smooth muscle”. The original version of this work was submitted to *Biochemical Pharmacology*; but unfortunately, following peer-review, it was not accepted for publication. A revised version of this manuscript was prepared and submitted to *Life Sciences*. This work was also peer-reviewed and is awaiting confirmation on its acceptance for publication.

Declaration of co-authorship and contribution

Full bibliographic details and Status

Irwin JC, Fenning AS & Vella RK. Statins with different lipophilic indices exert distinct effects on skeletal, cardiac and vascular smooth muscle. Manuscript submitted for publication.
Under review

Nature of Candidate's Contribution, including percentage of total

JC Irwin (85%): Study conceptualisation and design, acquisition of data, analysis and interpretation of data, writing of original draft, critical revision of manuscript and approval of final version.

Nature of all Co-Authors' Contributions, including percentage of total

AS Fenning (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version. RK Vella (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Abstract

Aims: Data concerning the influence of statin lipophilicity on the myotoxic and pleiotropic effects of statins is conflicting, and mechanistic head-to-head comparison studies evaluating this parameter are limited. In order to address the disparity, this mechanistic investigation aimed to assess the effects of two short-acting statins with different lipophilic indices on skeletal, cardiac and vascular smooth muscle physiology.

Materials and Methods: Young female Wistar rats were randomised to simvastatin (80 mg kg^{-1} day^{-1}), pravastatin (160 mg kg^{-1} day^{-1}) or control treatment groups. Changes in functional muscle performance were assessed, as well as mRNA levels of genes relating to atrophy, hypertrophy, mitochondrial function and/or oxidative stress.

Key findings: There were no significant differences in the mRNA profiles of isolated skeletal muscles amongst the treatment groups. In terms of functional performance, simvastatin reduced functionality, but treatment with pravastatin significantly improved force production. Rodents given simvastatin demonstrated comparable myocardial integrity to the control group. Conversely, pravastatin reduced left ventricular action potential duration, diastolic stiffness and *Mhc-β* expression. Pravastatin improved endothelium-dependent relaxation, particularly in muscular arteries, but this effect was absent in the simvastatin-treated rats. The responsiveness of isolated blood vessels to noradrenaline also differed between the statin groups. The findings of this study support that the effects of statins on skeletal, cardiac and vascular smooth muscle vary with their lipophilic indices.

Significance: The results of this work have important implications for elucidating the mechanisms responsible for the myotoxic and pleiotropic effects of statins.

1. Introduction

The precise mechanisms and factors which contribute to the development of statin-induced myotoxicity remain to be fully elucidated [1]. Establishing these parameters, however, is imperative for developing more effective interventions for managing statin-associated muscle symptoms (SAMS), and thus improving patient compliance with these pharmaceuticals [2]. Certain formulations, pharmacokinetic profiles and doses of statins are associated with a higher frequency of skeletal muscle complaints [3]. In particular, lipophilic formulations are reported to have a greater myotoxic potential compared to their hydrophilic counterparts [4-6].

Lipophilic statins are defined as having a Log *P* partition coefficient > 0 and include atorvastatin, fluvastatin, lovastatin, simvastatin and pitavastatin [7, 8]. Conversely, hydrophilic statins, namely pravastatin and rosuvastatin, possess a Log *P* partition coefficient < 0 [7]. It has been suggested that, unlike lipophilic types, hydrophilic statins are not myotoxic as they cannot cross the cell membranes of non-hepatic cells [5, 9]. However, *in vitro* investigations have identified organic anion transporting polypeptides (OATPs) which facilitate the entry of hydrophilic statins into skeletal muscle where they can then exert damaging effects [10, 11].

Evidence from human studies concerning the role of lipophilicity on the frequency of SAMS is also conflicting. While higher rates of SAMS have been reported in individuals given lipophilic statins [12], other trials have found no difference in the frequency of adverse muscle-related events between lipophilic and hydrophilic variants [13, 14]. Several meta-analyses have also found no association between statin lipophilicity and SAMS [2, 15, 16]. Furthermore, reports from clinical practice describe comparable rates of muscle-related complaints amongst individuals taking hydrophilic statins and lipophilic alternatives [17-19].

Like skeletal muscle, the effects of statins on cardiac and vascular smooth muscle are also postulated to differ with their lipophilicity. However, while some studies report that lipophilic statins exert greater protective effects on cardiovascular parameters [13, 20, 21], others indicate that hydrophilic formulations confer superior benefits [12]. Moreover, there is evidence that the effects of statins on cardiovascular outcomes do not vary with lipophilicity [22]. Ultimately, the existing disparities in the literature highlight that more studies are needed in order to clarify the influence of lipophilicity on the physiological effects of statins. Addressing this point will not only assist in elucidating the mechanisms causing SAMS, but also the processes underlying the pleiotropic effects linked to these medications.

Accordingly, this investigation aimed to investigate the effects of statin lipophilicity on skeletal, cardiac and vascular smooth muscle function via a head-to-head comparative study of simvastatin (Log *P* = 4.7) and pravastatin (Log *P* = -0.2). These formulations were selected as they are both "short-acting" statins (i.e. they have short elimination half-lives) [23] and have often been compared in human studies [12, 13, 20, 24]. The simvastatin treatment regimen employed in this investigation has been shown to induce physiological changes in skeletal muscle which are characteristic of statin-induced myalgia [25]. Therefore, this study was also able to assess the changes which occur in cardiovascular performance in the presence of SAMS.

2. Materials and Methods

2.1 Ethical approval

All treatments and methods used in this investigation were approved by the Animal Ethics Committee of Central Queensland University (AEC: 20217), according to guidelines from the National Health and Medical Research Council of Australia (NHMRC). Rodents were housed at 22 ± 2^{o}C and provided with food and water *ad libitum*. Body mass, water intake and food intake were measured to monitor animal health during the treatment period.

2.2 Animals and treatment protocols

Young (12-week old) female Wistar rats (250-300 g) were obtained from the Central Queensland University Rodent Breeding Colony (Rockhampton) and randomised to one of three treatment groups: simvastatin 80 mg kg^{-1} day^{-1} (SIM80, n = 26), pravastatin 160 mg kg^{-1} day^{-1} (PRAV160, n = 26) or control (CON, n = 26). Simvastatin 80 mg and pravastatin 80 mg tablets were dissolved in a 10% v/v solution of Polysorbate20 in milli-Q water and delivered daily via oral gavage for two weeks [25]. Owing to the pharmacodynamic resistance of rats to statins (as well as differences in rates of metabolism), high doses of these pharmaceuticals are required in order to induce physiological effects which resemble that observed in humans [26-28]. The doses of simvastatin and pravastatin employed in the present study were selected in a 1:2 mg ratio (i.e. 80 mg versus 160 mg) as they are suggested to be equipotent when administered in this ratio [29, 30]. This investigation was conducted using female rodents as this sex is more appropriate for studying the pathogenesis of SAMS in rodents [25].

2.3 Biometric assessments

Following two weeks of treatment, rodents were euthanised via a 1.0 mL intraperitoneal injection of sodium pentobarbitone (187.5 mg mL^{-1}). Death was confirmed by a lack of

responses (pedal and corneal reflexes). Gastrocnemius, soleus and tibialis anterior muscles, as well as the left ventricle, were isolated, weighed and stored at -80^{o}C for molecular analyses.

2.4 Ex vivo assessment of skeletal muscle performance

Electrical field stimulation was used to establish force-frequency curves (FFCs) for isolated skeletal muscles [25]. Tissues were transferred to warmed (37^{o}C) organ baths containing gassed (oxygen (O_2) 95% / carbon dioxide (CO_2) 5%) modified Krebs-Henseleit buffer (KHB, all in mM concentrations: sodium chloride 119, potassium chloride 5, magnesium sulfate 1, potassium dihydrogen phosphate 1, sodium hydrogen carbonate 25, glucose 11 and calcium chloride 2; pH ~ 7.4). Muscles were attached to a glass hook suspended between two platinum zigzag electrodes and stimulated at increasing frequencies between 1 to 100 Hz (output 100 V for 5 seconds every 135 seconds). Non-linear regression of normalised FFCs – up to 100 Hz, 40 Hz and 70 Hz for gastrocnemius, soleus and tibialis anterior muscles, respectively – was performed to calculate Log_{10}EF50 values (i.e. the frequency required to obtain half-maximal response). The muscles selected for this study have variable ratios of oxidative to glycolytic fibers (soleus > tibialis anterior > gastrocnemius) and this enabled the fiber-selective effects of statins on skeletal muscle performance to be investigated.

2.5 Ex vivo assessment of left ventricular compliance

The Langendorff heart preparation was performed (n = 13 per group) according to established protocols [31]. Isolated hearts were cannulated via the aorta and perfused with warmed (37^{o}C), gassed (O_2 95% / CO_2 5%) modified KHB. A latex balloon was inserted into the left ventricle and inflated to obtain increasing increments of diastolic pressure (0 mmHg to 30 mmHg).

Hearts were paced at 250 bpm by electrode stimulation of the right atrium for the entirety of the experiment. Data pertaining to maximum +d*P*/d*t* (rate of contraction), maximum -d*P*/d*t* (rate of relaxation), end systolic pressure, developed pressure and coronary flow was obtained at a pressure of 10 mmHg. Diastolic stiffness was calculated as per the computations described by Jackson et al. [32].

2.6 Single cell microelectrode analysis of left ventricular electrophysiology

Isolated left ventricular papillary muscles (*n* = 13 per group) were immersed in warmed (37^{o}C), gassed (O_2 95% / CO_2 5%) Tyrode's physiological salt solution (TPSS, all in mM concentrations: sodium chloride 137, potassium chloride 5, magnesium chloride 1, sodium dihydrogen phosphate 0.4, sodium bicarbonate 23, calcium chloride 2, glucose 6, ascorbic acid 0.3 and ethylenediaminetetraacetic acid 0.1; pH ~ 7.4) and fixed between two platinum electrodes [32]. The superior end of the papillary muscles was secured to a modified SensoNor AE 801 micro-force transducer using a stainless-steel hook. Contractions were induced with electrical field stimulation (frequency of 1 Hz, pulse width of 0.5 msec and stimulus strength 20% above threshold), then the muscles were impaled with a potassium chloride (KCl)-filled microelectrode. Bioelectrical activity was recorded to measure the following parameters: resting membrane potential (RMP), action potential amplitude (APA), action potential duration at 20%, 50% and 90% repolarisation (APD20, APD50 and APD90), force of contraction (FC) as well as time to 90% relaxation (TR90). Isolated hearts could not be used for both the Langendorff and microelectrode experiments so were randomly allocated to either preparation (*n* of 12 for the Langendorff and *n* of 14 for the microelectrode studies).

2.7 Vascular reactivity in isolated elastic and muscular arteries

Segments of thoracic aortas and mesenteric arteries were placed in organ baths containing warmed, gassed (O_2 95% / CO_2 5%) TPSS. Aortas were set to a resting tension of 10 mN and mesenteric arteries were normalised to a transmural pressure of 100 mmHg [32]. Responses to cumulative concentrations of acetylcholine, sodium nitroprusside and noradrenaline (ranging between 1 x 10^{-9} M and 3 x 10^{-4} M) were recorded. Concentration-response curves (CRCs) were assessed using non-linear regression to calculate $Log_{10}EC50$ values. For analysis of relaxation responses, the blood vessels were first constricted with a sub-maximal concentration of noradrenaline (1 x 10^{-6} M for thoracic aortas and 1 x 10^{-5} M for mesenteric arteries) and allowed to reach a stable plateau before the CRCs were initiated. Endothelium-dependent and -independent relaxation responses are expressed as percentage relaxation of tone induced by noradrenaline.

2.8 Quantitative reverse transcription PCR (RT-qPCR)

mRNA was extracted from tissues using Trizol™ and reverse-transcribed to cDNA with Superscript™ III reverse transcriptase, as per the manufacturer's instructions (Applied Biosystems Inc.). Taqman™ gene expression assays (Applied Biosystems Inc.) (**Table 1**) and Rotor-Gene Q equipment (Qiagen) were used for the PCR assays, and all analyses were performed according to the manufacturers' protocols. Samples with *Ct* values > 35 were excluded. No-RT controls were acceptable if *Ct* values were $\geq$ 5 cycles compared to samples [33]. mRNA levels were normalised to *Gapdh* for calculation of relative expression, as per the delta-delta C*t* method [34].

2.9 Data and statistical analysis

Prior to statistical testing, ROUT analysis was used to identify potential outliers. Datasets were tested for normality using the D'Agostino-Pearson test or Kolomogov-Smirnov test (depending on sample size). Statistically significant differences were assessed using one-way ANOVA, or repeated-measures two-way ANOVA, followed by Tukey post-hoc testing as appropriate. Nonparametric results were analysed using the Kruskal-Wallis test with Dunn's post-hoc testing. Statistical significance was set at an alpha level of 0.05. Data are presented as means (SD) and all analyses were conducted using GraphPad Prism 8 (GraphPad Software, La Jolla, USA).

2.10 Reagents and chemicals

The buffers used in this study were made using analytical-grade chemicals purchased through Thermofisher Scientific (Scoresby, Australia). Acetylcholine (≥ 99%), sodium nitroprusside (≥ 99%) and noradrenaline (≥ 99%) were purchased from Merck (Castle Hill, Australia).

3. Results

3.1 Skeletal muscle force production

As two-way ANOVA showed significant interaction for all FFCs, simple effects were assessed. In comparison to both the CON and PRAV160 groups, gastrocnemius muscles isolated from SIM80 rats showed reduced force production from 60 Hz onwards with statistically significant differences at 90 and 100 Hz ($P < 0.05$) (**Fig. 1**). Correspondingly, one-way ANOVA of

maximum force production identified significantly lower performance in the SIM80 rats versus the CON animals ($P < 0.02$) (**Table 2**). However, within group variability negated any statistical significance in comparison to the PRAV160 group ($P = 0.07$). $Log_{10}EF50$ values were significantly higher in the CON and PRAV160 groups in comparison to the SIM80 rats ($P < 0.0001$). Additionally, $Log_{10}EF50$ values were significantly higher in the PRAV160 animals compared to the CON group ($P < 0.0001$).

There were no significant differences in FFCs, maximum force production or $Log_{10}EF50$ values between the CON and SIM80 groups (**Fig 1** and **Table 2**). Conversely, treatment with pravastatin significantly improved skeletal muscle performance. Specifically, PRAV160 rats exhibited greater force production compared to the CON and SIM80 animals between 10 to 40 Hz and 5 to 40 Hz, respectively ($P < 0.05$). Correspondingly, maximum force production was significantly higher in the PRAV160 group compared to the CON and SIM80 animals ($P < 0.0001$), but there was no difference in $Log_{10}EF50$ values.

Treatment with pravastatin also increased force production in the tibialis anterior. Statistically significant differences in performance were observed from 60 and 70 Hz onwards in comparison to the CON and SIM80 groups, respectively ($P < 0.05$) (**Fig 1**). One-way ANOVA of maximum force production identified a significant difference between the CON and PRAV160 groups ($P = 0.04$), but within-group variability negated any statistical significance between the PRAV160 and SIM80 animals ($P = 0.06$) (**Table 2**). $Log_{10}EF50$ values were significantly higher in the tibialis anterior muscles isolated from the CON and PRAV160 rats compared to the SIM80 group ($P = 0.04$ and < 0.0001, respectively). Additionally, $Log_{10}EF50$ values were also significantly higher in the PRAV160 animals compared to the CON rats ($P < 0.0001$).

3.2 Skeletal and cardiac muscle mass

There were no differences in the mass of isolated gastrocnemius, soleus or tibialis anterior muscles amongst any of the treatment groups (**Table 3**). Left ventricle mass was similar between the CON and PRAV160 group but was significantly increased in the SIM80 animals (P = 0.02 vs. CON and P = 0.007 vs. PRAV160) (**Table 3**).

3.3 mRNA expression profiles in skeletal muscle

There were no significant differences in the expressions of *Atrogin-1*, *Mstn*, *Tnfα*, *Pgc-1α*, *Pdk4*, *Pparα*, *Nox2* or *Sod1* in isolated gastrocnemius muscles (**Fig. 1**). *Mt1a* mRNA levels in the both the SIM80 and PRAV160 rats did not differ significantly relative to the CON group. However, *Mt1a* expression in the SIM80 animals was slightly higher (approximately 3-fold) compared to the PRAV160 group (P = 0.01).

In the soleus, the mRNA levels of *Atrogin-1*, *Mstn*, *Tnfα*, *Pdk4*, *Pparα* and *Mt1a* were similar amongst the treatment groups (**Fig. 1**). *Pgc-1α* expression was slightly reduced (approximately 2-to-3 fold) in both statin-treated groups relative to the CON animals, however this effect was only statistically significant for the PRAV160 group (P = 0.03). Although slightly reduced in the PRAV160 rats, *Nox2* demonstrated no significant difference in expression amongst the treatment groups (P = 0.08). *Sod1* was mildly reduced in the PRAV160 animals and this effect was statistically significant in comparison to the CON group (P = 0.03 vs. CON and P = 0.09 vs. SIM80).

There were no significant differences in the expression of *Atrogin-1*, *Mstn*, *Pdk4* or *Pparα* in tibialis anterior muscles (**Fig. 1**). The mRNA levels of *Tnfα* were similar between the CON and PRAV160 groups, but expression was slightly increased in the SIM80 group relative to the CON group ($P = 0.01$). *Pgc-1α* expression was similar between the SIM80 and CON groups, but mildly reduced in the PRAV160 animals. This change was statistically significant in comparison to the SIM80 group ($P = 0.03$). Average *Mt1a*, *Nox2* and *Sod1* expression appeared to be mildly increased by SIM80 treatment (approximately 2-to-3-fold). Nonetheless, these effects were not statistically significant as the increased average could be attributed to one or two high individual values (these values had not been identified as outliers by the ROUT analysis owing to large within-group variability). *Nox2* mRNA levels were slightly reduced by PRAV160 treatment (near 3-fold), but this was not a statistically significant effect.

3.4 Left ventricular pump function

There was no evidence of altered left ventricular performance in either the SIM80 or PRAV160 rats (**Table 4**). Values for maximum +dP/dt, maximum -dP/dt, end systolic pressure, developed pressure and coronary flow were similar amongst the treatment groups. There was no difference in diastolic stiffness between the SIM80 and CON groups, however values were significant reduced in the PRAV160 animals ($P = 0.02$ vs. CON and $P = 0.003$ vs. SIM80).

3.5 Left ventricular electrophysiology

RMP and APA were not significantly altered by either simvastatin or pravastatin administration (**Table 4**). APD20 and APD50 were similar between the CON and SIM80 animals but were significantly shortened in the PRAV160 group ($P < 0.02$). APD90 was slightly prolonged in

the SIM80 rats relative to the CON group, however this effect was not significant. APD90 was reduced in PRAV160 animals and this effect was statistically significant in comparison to the SIM80 group (P =0.04). FC was slightly increased in the statin-treated groups (CON < SIM80 < PRAV160), however no statistically significant differences were identified. TR90 was decreased in both statin-treated groups, but this effect was only statistically significant for the SIM80 rats (P = 0.047).

3.6 mRNA expression profile in isolated left ventricles

There were no significant differences in the expression of *Bnp* amongst the treatment groups (**Fig 2**). There was a slight reduction in the mRNA level of *Mhc-β* in the PRAV160 rats, but this change was only statistically significant in comparison to the SIM80 rats (P = 0.06 vs. CON and P = 0.01 vs. SIM80).

3.7 Blood vessel reactivity

As all CRCs showed significant interaction, simple effects were analysed. There were no differences in endothelium-independent relaxation for the isolated thoracic aortas (**Fig. 2**). $Log_{10}EC50$ values for acetylcholine, however, were significantly lower in the SIM80 animals compared to the CON and PRAV160 rats (P = 0.001 vs. CON and P < 0.0001 vs. PRAV160) (**Table 5**). Endothelium-dependent relaxation was also largely similar amongst the treatment groups. There was a slight improvement in the PRAV160 animals, but this effect was only statistically significant at 3×10^{-6} M relative to the SIM80 rats (P = 0.02). Accordingly, there were no differences in maximum relaxation or $Log_{10}EC50$ values. PRAV160 rats exhibited significantly lower noradrenaline-induced contraction responses compared to the CON and

SIM80 animals from 1 x 10^{-8} M to 3 x 10^{-7} M and 1 x 10^{-7} M to 3 x 10^{-7} M, respectively (P < 0.05). There were no significant differences in responses by 1 x 10^{-6} M, however, contraction was significantly greater in the PRAV160 animals at the final concentration of 1 x 10^{-5} M (P = 0.002 vs. CON and P = 0.04 vs. SIM80). The Log_{10}EC50 for noradrenaline was significantly increased in the PRAV160 animals (P < 0.0001 vs. CON and P = 0.002 vs. SIM80). Within-group variability, however, negated any statistically significant difference for the one-way ANOVA of maximum contraction.

In isolated mesenteric arteries, endothelium-dependent relaxation was greatest in the PRAV160 rats for most of the CRC (P < 0.05) (**Fig. 3**). Accordingly, maximum relaxation was significantly increased in these animals (P = 0.0008 vs. CON and P = 0.04 vs. SIM80) (**Table 5**). There was no difference in acetylcholine Log_{10}EC50 between the CON and PRAV160 rats, but both groups showed significantly lower values compared to the SIM80 animals (P < 0.0001). Endothelium-independent relaxation in mesenteric arteries was slightly reduced in PRAV160 animals at the start of the CRC, with statistically significant differences in comparison to the SIM80 group at 1 x 10^{-8} M and 3 x 10^{-8} M (P < 0.05). Congruently, sodium nitroprusside Log_{10}EC50 was highest in the PRAV160 rats, and this was statistically significant in comparison to the SIM80 group (P = 0.0001 vs. SIM80 and P = 0.06 vs. CON). There was no difference in sodium nitroprusside Log_{10}EC50 between the SIM80 and CON groups. Endothelium-independent relaxation was similar amongst all the groups from 1 x 10^{-7} M onwards, and no differences in maximum responses were observed. Mesenteric arteries isolated from PRAV160 animals exhibited reduced contraction at the lower doses of the CRC (1 x 10^{-6} M to 1 x 10^{-5} M) compared to the other treatment groups (P < 0.05) (**Fig. 3**). Correspondingly, the noradrenaline Log_{10}EC50 was significantly higher in the PRAV160 animals relative to CON and SIM80 rats (P < 0.0001). Log_{10}EC50 was also increased in the

SIM80 group relative to the CON animals ($P = 0.002$). Nonetheless, both statin-treated groups exhibited significantly greater contraction compared to the CON group at higher doses of the CRC ($P < 0.05$). Accordingly, maximum responses to noradrenaline were significantly higher in the SIM80 and PRAV160 rats relative to the CON animals ($P = 0.03$ vs. SIM80 and $P = 0.02$ vs. PRAV160).

4. Discussion

The findings of this comparative rodent-based study demonstrate that the myotoxic potential of statins with different lipophilic indices varies significantly. Overall, treatment with simvastatin significantly reduced skeletal muscle performance (namely in the gastrocnemius), while pravastatin improved functionality (most notably in the soleus and tibialis anterior). The onset of muscle fatigue in the SIM80 group was exemplified by a leftward shift in the gastrocnemius FFC [35] and loss of force production. Conversely, the PRAV160 animals demonstrated a significant increase in the $Log_{10}EF50$ for the gastrocnemius FFC. Although this shift was not accompanied by an increase in muscle force production, it may still indicate a reduced propensity to fatigue [35].

Pravastatin treatment increased force production in the soleus, however, this was not accompanied by a significant change in the $Log_{10}EF50$. Similarly, although the administration of simvastatin and pravastatin had differential effects on force production, both treatment groups exhibited a rightward shift in the FFC. These findings indicate that the relationship between force production and FFC-shift may differ with the fiber-type composition of skeletal muscle. More pertinently, the results demonstrate a fiber-selective trend in the physiological

effects of statins. Specifically, simvastatin exerted its greatest myotoxic effects in the predominately fast-twitch glycolytic gastrocnemius, an observation which is common in studies of SAMS [24, 25, 36]. Conversely, pravastatin induced the largest improvement in muscle performance in muscles with comparatively more slow-twitch oxidative fibers (i.e. the soleus and tibialis anterior).

In order to further evaluate the fiber-selective changes induced by statin treatment, the mRNA levels of genes typically associated with altered skeletal muscle integrity were assessed. While statistically significant differences in expression were observed, the actual changes in mRNA levels were quite mild. Moreover, the alterations in gene expression were not consistent with skeletal muscle performance. Indeed, although the SIM80 and PRAV160 groups exhibited considerable differences in muscle performance, the mRNA expression profiles were largely comparable. Conversely, *Tnfα* expression was significantly elevated in the SIM80 tibialis anterior muscles relative to the CON animals, but there was no difference in force production between these groups. Statin-induced alterations in muscle integrity are typically associated with changes in mitochondrial biogenesis, metabolism, protein degradation, oxidative stress and/or inflammation [37-39]. The lack of correlation between muscle function and the expression of genes related to these processes in the present study, however, support that other factors may underlie the effects of statins on skeletal muscle force production [40].

Outwardly, the results of this study corroborate other reports that lipophilic statins cause skeletal myotoxicity more readily than pravastatin [4-6, 41, 42]. However, they disagree with the perception that pravastatin cannot induce significant physiological effects in skeletal muscle [6, 42, 43]. To the best of our knowledge, this is the first study to report improved skeletal muscle performance in rats following the administration of pravastatin. The absence

of any significant effect of pravastatin on muscle physiology in other rodent-based studies may be related to the lower doses used in these investigations (e.g. 8-55 mg/kg). It is well-established that, owing to pharmacokinetic and pharmacodynamic differences, rodents require much larger doses of statins to exhibit changes in skeletal muscle physiology which resemble that observed in humans [26, 27]. While statin therapy can be associated with SAMS, instances of improved muscle strength following treatment with these pharmaceuticals has been reported [44]. Hence, the PRAV160 treatment regimen used in this study provides a suitable platform through which the mechanisms underlying this effect could be investigated. Further mechanistic studies comparing the intramuscular changes induced by the PRAV160 dosing protocol versus the SIM80 SAMS model will be useful for elucidating the factors responsible for statin-induced myotoxicity. In particular, excitation-contraction coupling parameters (such as sarcoplasmic reticulum calcium release and intramuscular ATP levels) should be examined in order to expound the alterations in muscle force generation reported in this investigation.

The presence of myalgia in the SIM80 group, but absence in the PRAV160 animals, allowed for potential changes in cardiac and vascular integrity during SAMS to be examined. In terms of the myocardium, there was a slight (albeit statistically significant) increase in the mass of left ventricles isolated from the SIM80 rats. This change, however, was not accompanied by an increase in *Mhc-β* or *Bnp* mRNA levels. Moreover, there was no prolongation of TR90 or significant increase in APD in the SIM80 animals relative to the CON group, otherwise hallmark features of hypertrophied hearts [45, 46]. Accordingly, these results indicate that the observed increase in left ventricular mass was not biologically significant. This point is further exemplified by the absence of any significant differences in left ventricular compliance between the SIM80 and CON groups. Together, these findings suggest that statin-induced myalgia is not associated with major alterations in myocardial integrity.

Overall, the effect of pravastatin on cardiac muscle varied from that of simvastatin. In particular, the left ventricles isolated from the PRAV160 rats demonstrated lower *Mhc-β* mRNA expression and diastolic stiffness relative to the other treatment groups. APD was also significantly reduced following treatment with pravastatin, suggesting a potential alteration in the sodium-calcium exchange current [47]. As no changes in systolic or diastolic function were observed in the PRAV160 animals, the clinical significance of the aforementioned changes is unclear. Singularly, these factors have been associated with improved left ventricular remodelling and cardiac performance in pathological conditions [48-50]. Therefore, it would be beneficial to repeat this study using a rodent model of cardiovascular disease in order to validate the biological significance of these changes.

Endothelium-independent relaxation in thoracic aortas was comparable across the treatment groups. Variation in group $Log_{10}EC50$ values for sodium nitroprusside were observed, however these differences did not translate to any clinically significant alterations in functional performance. Similarly, while the PRAV160 animals demonstrated reduced responsiveness to sodium nitroprusside at the lower doses of the CRC, maximum relaxation was the same amongst all treatment groups. Ultimately, the absence of any considerable difference in relaxation suggests that, akin to the myocardium, vascular smooth muscle performance is not adversely affected during statin-induced myalgia.

The administration of pravastatin increased endothelium-dependent relaxation in isolated blood vessels, particularly in the mesenteric arteries. This improvement may reflect an increase in endothelial nitric oxide synthase (eNOS) expression/activity which has previously been observed in statin-treated subjects [52, 52]. Unlike the PRAV160 rats, the SIM80 animals did

not exhibit an improvement in endothelial performance. This result contrasts with reports of enhanced endothelium-dependent relaxation following simvastatin treatment in studies using rodent models of cardiovascular disease [53, 54]. As endothelial dysfunction was absent in the animals used in the present investigation (i.e. rodents were young and healthy), it is pertinent to re-perform this investigation using older rats in order to assess whether the superior effects of pravastatin on endothelial function are maintained.

Although noradrenaline $Log_{10}EC50$ values were altered in both statin-treated groups, only the PRAV160 rats exhibited differences in functional performance which were consistent with these changes. Specifically, the elastic and muscular arteries from these animals demonstrated a reduced responsiveness to noradrenaline at the lower doses of the CRC. As denuded vessels were not used in this study, it is possible that the increased presence of endothelial-derived relaxation factors (as exemplified by the improvement in endothelium-dependent relaxation) may have contributed to the delay in vasocontraction [55]. Despite this effect, the PRAV160 rats demonstrated significantly higher maximum noradrenaline-induced contraction compared to the CON group in both blood vessel types. While unchanged in the thoracic aortas, contractile performance was also increased in the mesenteric arteries isolated from the SIM80 rats. Contrary to these results, the vasoprotective effects of statins are typically associated with a reduction in agonist-induced contractile responses [56, 57]. As the PRAV160 and SIM80 rats exhibited no signs of impaired cardiovascular performance, the enhancement of noradrenaline-induced contraction in this study is not considered to be pathological. Nonetheless, further investigations are required to elucidate the mechanisms underlying these changes in vascular function (and their potential to be altered in the presence of cardiovascular disease).

5. Conclusion

The findings of this work demonstrate that the effects of simvastatin and pravastatin on skeletal, cardiac and vascular smooth muscle differ significantly. Hence, these results support that the physiological effects of statins vary with their lipophilic index. It should be noted that while the influence of dose and elimination half-life were controlled for in this study, other chemical properties (such as the extent of protein-binding) may have influenced the responses observed in this investigation. Therefore, further studies using a larger selection of lipophilic and hydrophilic statins are required in order validate the findings of this work. In any case, the results of this investigation are significant as they demonstrate that pravastatin can cause considerable physiological changes in muscle integrity. The findings obtained from this study have important implications for characterising the potential myotoxic and pleiotropic effects of statins.

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Figure Legend

Fig. 1 Normalised force-frequency response curves and mRNA expression in muscles isolated from control (CON) and statin-treated (SIM80 and PRAV160) rats: (A) and (D) shows results for gastrocnemius muscles; (B) and (E) soleus muscles and; (C) and (F) tibialis anterior muscles. Results for the force-frequency curves are expressed as means with standard deviations (*n* of 18-26 per group following removal of inviable tissues and outliers by ROUT analysis). This data was analysed using two-way repeated measures ANOVA followed by Tukey post-hoc test. Results for mRNA expression are expressed as mean Log_2 fold changes with standard deviations (*n* of 5-6 per group following removal of outliers by ROUT analysis). This data was analysed using one-way ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and $^{\#}$ indicates $P < 0.05$ versus SIM80.

Fig. 2 mRNA expression in left ventricles isolated from control (CON) and statin-treated (SIM80 and PRAV160) rats. Results are expressed as mean Log_2 fold changes with standard deviations (*n* of 6 per group following removal of outliers by ROUT analysis). This data was analysed using one-way ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and $^{\#}$ indicates $P < 0.05$ versus SIM80.

Fig. 3 Concentration response curves in thoracic aortas (A-C) and mesenteric arteries (D-F) in muscles isolated from control (CON) and statin-treated (SIM80 and PRAV160) rats: (A) and (D) shows results for acetylcholine; (B) and (E) sodium nitroprusside and; (C) and (F) noradrenaline. Results are expressed as means with standard deviations (*n* of 18-26 or 17-25 per group for aortas and mesenteric arteries, respectively, following removal of inviable tissues and outliers by ROUT analysis). This data was analysed using two-way repeated measures

ANOVA followed by Tukey post-hoc test. Relaxation responses were normalised to percentage relaxation of maximum noradrenaline-induced precontraction. * indicates $P < 0.05$ versus CON and # indicates $P < 0.05$ versus SIM80.

Table 1 Gene expression assays

Gene of interest	Taqman™ Gene Expression Assay	Amplicon length
Mitochondrial biogenesis		
Ppargc1α (*Pgc-1α*)	Rn00580241_m1	94
Atrophy		
Fbxo32 (*Atrogin-1*)	Rn00591730_m1	61
Mstn	Rn00569683_m1	67
Metabolism		
Pdk4	Rn00585577_m1	76
Pparα	Rn00566193_m1	98
Oxidative stress		
Cybb (*Nox2*)	Rn00576710_m1	77
Sod1	Rn00566938_m1	62
Mt1a	Rn00821759_g1	88
Hypertrophy		
Mhc-β	Rn01488777_g1	76
Bnp	Rn00580641_m1	106

Table 2 $Log_{10}EF50$ and maximum force production values of gastrocnemius, soleus and tibialis anterior muscles isolated from control (CON) and statin-treated (SIM80 and PRAV160) rats.

Parameter	CON	SIM80	PRAV160
Gastrocnemius $Log_{10}EF50$	1.47(0.25)	1.28(0.37)*	1.61(0.15)*#
Soleus $Log_{10}EF50$	0.79(0.58)	0.88(0.40)	0.82(0.35)
Tibialis anterior $Log_{10}EF50$	0.89(0.40)	0.98(0.58)*	1.29(0.23)*#
Gastrocnemius Maximum force (g/cm^2)	13.25(3.39)	10.12(4.46)*	12.76(2.84)
Soleus Maximum force (g/cm^2)	148.90(72.76)	138.90(89.45)	279.40(42.98)*#
Tibialis anterior Maximum force (g/cm^2)	18.78(9.06)	18.99(8.61)	25.29(7.47)*

Results are expressed as means with standard deviations. *n* of 18-26 per group for maximum force following removal of inviable tissues and outliers by ROUT analysis. Data was analysed using one-way ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and # indicates $P < 0.05$ versus SIM80.

Table 3 Mass of skeletal muscles and left ventricles isolated from control (CON) and statin-treated (SIM80 and PRAV160) rats.

Parameter	CON	SIM80	PRAV160
Gastrocnemius (mg gram body mass^{-1})	5.77(0.35)	5.81(0.47)	5.89(0.33)
Soleus (mg gram body mass^{-1})	0.52(0.05)	0.53(0.08)	0.54(0.06)
Tibialis anterior (mg gram body mass^{-1})	1.84(0.16)	1.87(0.12)	1.89(0.17)
Left ventricle (mg gram body mass^{-1})	2.32(0.33)	2.58(0.40)*	2.27(0.30)$^{\#}$

Results are expressed as means with standard deviations. *n* of 22-26 per group following removal of outliers by ROUT analysis. Data was analysed using one-way ANOVA followed by Tukey post-hoc test. *indicates $P < 0.05$ versus CON and $^{\#}$ indicates $P < 0.05$ versus SIM80.

Table 4 Parameters of left ventricular compliance and electrophysiology in control (CON) and statin-treated (SIM80 and PRAV160) rats.

Parameter	CON	SIM80	PRAV160
Left ventricular compliance			
Max +dP/dt (mmHg s^{-1})	2397(534.80)	2475(272.30)	2475(366.30)
Max –dP/dt (mmHg s^{-1})	-1449(392.90)	-1495(274.00)	-1500(201.10)
End systolic pressure (mmHg)	106.30(23.48)	111.60(17.47)	119.80(14.27)
Diastolic stiffness (κ)	33.46(8.25)	35.73(5.22)	25.87(4.49)*#
Developed pressure (mmHg)	96.75(23.62)	101.90(18.07)	110.1(14.31)
Coronary flow (mL)	10.68(1.29)	11.37(2.61)	11.19(2.75)
Left ventricular electrophysiology			
Resting membrane potential (mV)	-54.87(7.74)	-60.30(12.00)	-64.17(15.60)
Action potential amplitude (mV)	65.96(17.81)	59.93(15.13)	58.15(14.02)
Action potential duration 20% (ms)	17.45(5.26)	18.24(5.40)	12.18(1.86)*#
Action potential duration 50% (ms)	28.57(8.25)	31.36(11.57)	18.13(3.31)*#
Action potential duration 90% (ms)	79.67(28.30)	93.00(33.50)	66.93(14.10)#
Force of contraction (mN)	0.80(0.47)	0.91(0.68)	0.99(0.63)
Time to 90% relaxation (ms)	206.10(60.01)	159.00(52.23)*	166.70(34.98)

Results are expressed as means with standard deviations. *n* of 10-12 per group for Langendorff parameters and *n* of 13-14 per group for electrophysiology parameters following removal of inviable tissues and outliers by ROUT analysis. Data was analysed using one-way ANOVA followed by Tukey post-hoc test. dF/dt, rate of change in force; +dP/dt, maximum rate of contraction; -dP/dt, maximum rate of relaxation.[*] indicates $P < 0.05$ versus CON and [#] indicates $P < 0.05$ versus SIM80.

Table 5 $Log_{10}EC50$ and maximum responses in thoracic aortas and mesenteric arteries isolated from control (CON) and statin-treated (SIM80 and PRAV160) rats.

Parameter	CON	SIM80	PRAV160
Thoracic aortas			
Acetylcholine $Log_{10}EC50$	-6.72(1.03)	-6.75(1.01)	-6.75(0.66)
Sodium nitroprusside $Log_{10}EC50$	-7.83(0.68)	-7.98(0.56)*	-7.77(0.36)#
Noradrenaline $Log_{10}EC50$	-6.73(2.04)	-6.63(1.69)	-6.26(0.54)*#
Acetylcholine maximum relaxation (% relaxation)	41.30(19.04)	38.89(14.80)	43.52(14.89)
Sodium nitroprusside maximum relaxation (% relaxation)	46.98(16.65)	44.49(14.64)	47.39(14.80)
Noradrenaline maximum contraction (mN)†	3.78(1.99)	3.87(1.74)	4.18(2.36)
Mesenteric arteries			
Acetylcholine $Log_{10}EC50$	-7.24(1.41)	-6.45(0.94)*	-7.25(0.67)#
Sodium nitroprusside $Log_{10}EC50$	-7.01(1.18)	-7.19(0.98)	-6.80(0.75)#
Noradrenaline $Log_{10}EC50$	-5.55(0.90)	-5.37(0.59)*	-4.94(0.48)*#
Acetylcholine maximum relaxation (% relaxation)†	68.78(24.22)	76.47(20.17)	90.55(7.72)*#
Sodium nitroprusside maximum relaxation (% relaxation)	77.59(13.20)	82.49(13.31)	80.56(12.48)
Noradrenaline maximum contraction (mN)	6.68(3.23)	10.14(6.03)*	10.25(5.20)*

Results are expressed as means with standard deviations. *n* of 18-26 per group for aortas following removal of inviable tissues and outliers by ROUT analysis. *n* of 17-25 per group for mesenteric arteries following removal of inviable tissues and outliers by ROUT analysis. Data was analysed using one-way ANOVA followed by Tukey post-hoc test. * indicates $P < 0.05$ versus CON and # indicates $P < 0.05$ versus SIM80. † Data failed normality testing and was analysed using the Kruskal-Wallis test with Dunn's post-hoc test.

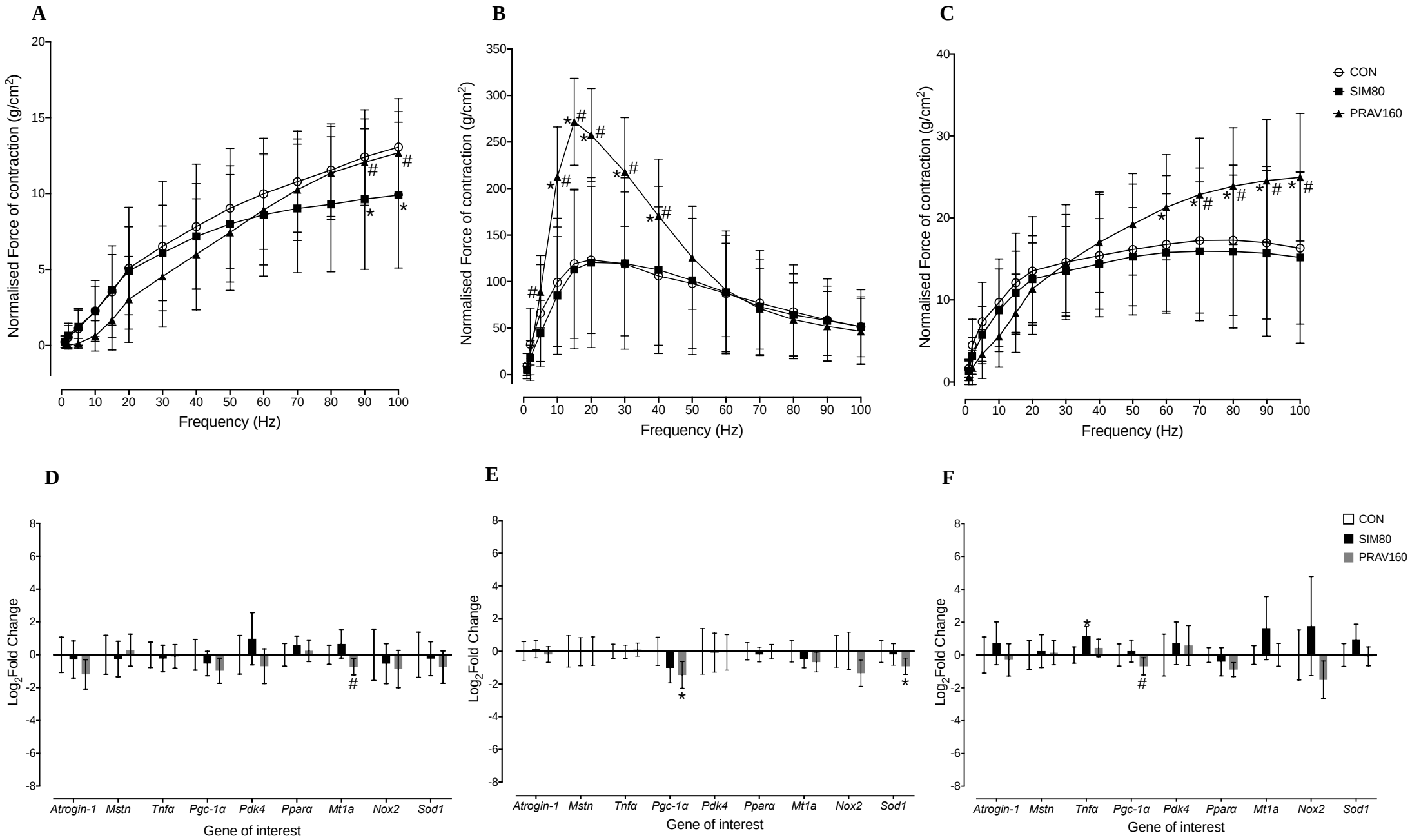
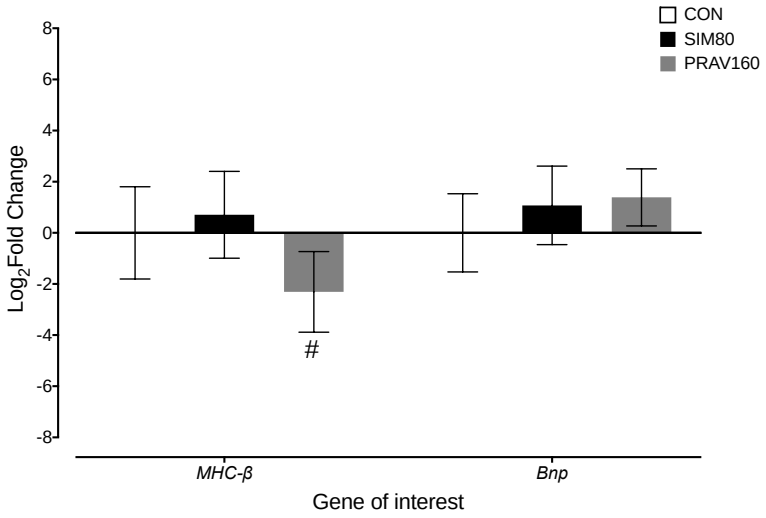

A
B
C
Normalised Force of contraction (g/cm2)
Frequency (Hz)
CON
SIM80
PRAV160
D
E
F
Log2Fold Change
Atrogin-1
Mstn
Tnfα
Pgc-1α
Pdk4
Pparα
Mt1a
Nox2
Sod1
Gene of interest

Fig 1.


CON
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8
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Gene of interest

Fig 2.

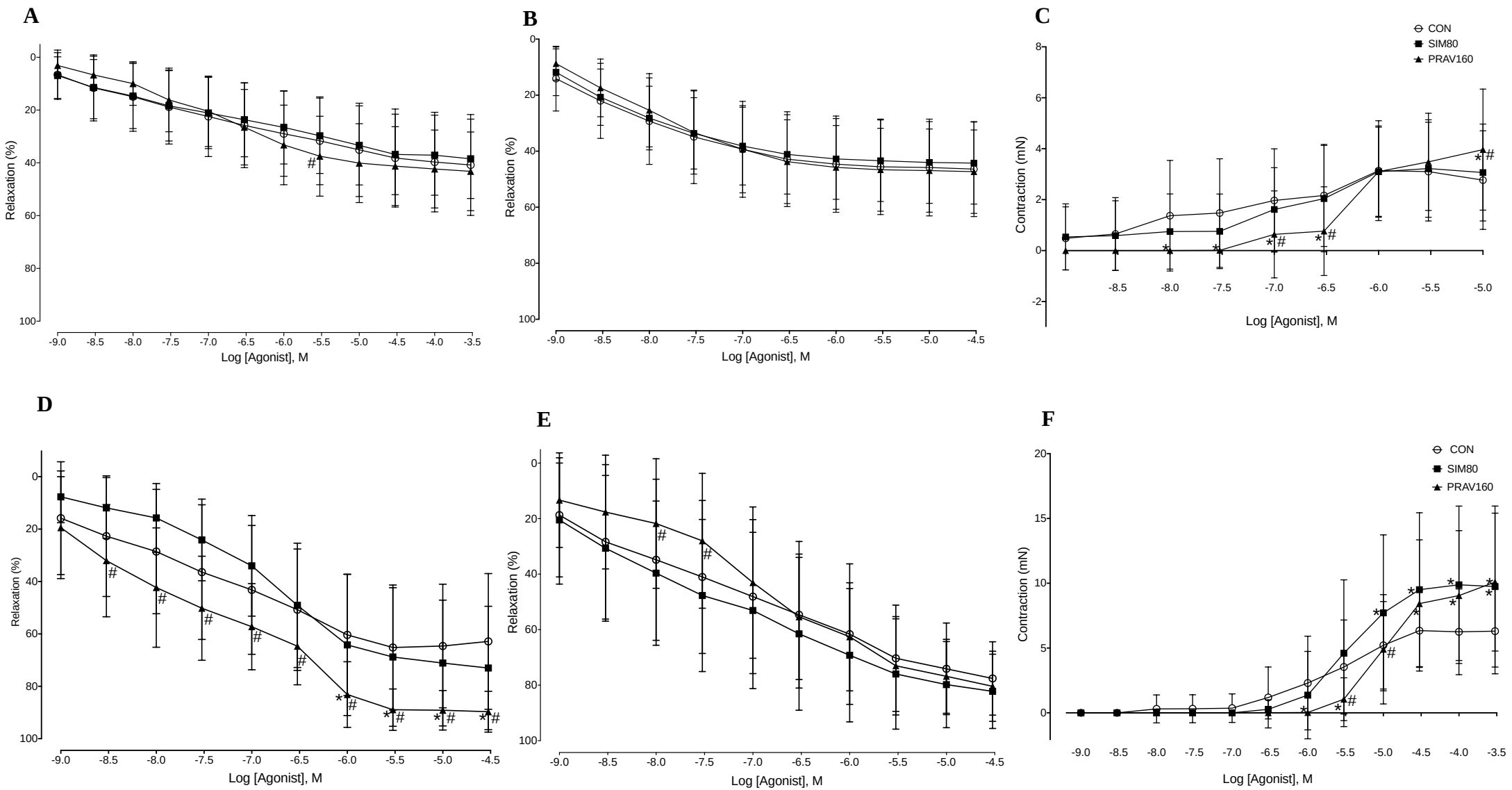
A
B
C
D
E
F
Relaxation (%)
Contraction (mN)
Log [Agonist], M
CON
SIM80
PRAV160

Fig 3.

CHAPTER 6

Geranylgeraniol prevents statin-induced skeletal muscle fatigue without causing adverse effects in cardiac or vascular smooth muscle performance

Preamble

Chapter 6 presents the results of a feasibility study which aimed to establish whether administering GGPP (in the form of geranylgeraniol) could prevent statin-induced myalgia. This investigation also sought to verify the effects of simvastatin on cardiovascular performance (as reported in the previous study), and thereby determine whether co-administration with geranylgeraniol altered these effects. As this was a feasibility study, it was not within the scope of this work to conduct a thorough analysis of the mechanisms underlying the physiological effects observed in this investigation. Nonetheless, the manuscript does suggest future research directions to elucidate the findings generated from this study.

This chapter contains a manuscript titled, “Geranylgeraniol prevents statin-induced muscle fatigue without causing adverse effects on cardiac or vascular smooth muscle performance”, which has been published in *Translational Research*.

Declaration of co-authorship and contribution

Full bibliographic details and Status

Irwin JC, Fenning AS, Vella RK. Geranylgeraniol prevents statin-induced myalgia without causing adverse effects in cardiac or vascular smooth muscle performance. Transl Res. Forthcoming 2019.
Published

Nature of Candidate's Contribution, including percentage of total

JC Irwin (85%): Study conceptualisation and design, acquisition of data, analysis and interpretation of data, writing of original draft, critical revision of manuscript and approval of final version.

Nature of all Co-Authors' Contributions, including percentage of total

AS Fenning (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version. RK Vella (7.5%): Study concept and design, acquisition of data, critical revision of manuscript and approval of final version.

Has this paper been submitted for an award by another research degree candidate (Co-Author), either at CQUniversity or elsewhere? (if yes, give full details)

No

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Geranylgeraniol prevents statin-induced skeletal muscle fatigue without causing adverse effects in cardiac or vascular smooth muscle performance

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The administration of geranylgeranyl pyrophosphate (GGPP) (or its precursor, geranylgeraniol (GGOH)) has been shown by several in vitro studies to be capable of abrogating statin-induced myotoxicity. Nonetheless, the potential of GGPP repletion to prevent statin-associated muscle symptoms (SAMS) in vivo is yet to be investigated. Therefore, this study aimed to evaluate the ability of GGOH to prevent SAMS in rodents. Female Wistar rats (12 weeks of age) were randomised to 1 of 4 treatment groups: control, control with GGOH, simvastatin or simvastatin with GGOH. Ex vivo assessment of force production was conducted in skeletal muscles of varying fiber composition. Ex vivo left ventricular performance and blood vessel function was also assessed to determine if the administration of GGOH caused adverse changes in these parameters. Statin administration was associated with reduced force production in fast-twitch glycolytic muscle, but coadministration with GGOH completely abrogated this effect. Additionally, GGOH improved the performance of muscles not adversely affected by simvastatin (ie, those with a greater proportion of slow-twitch oxidative fibers), and increased force production in the control animals. Neither control nor statin-treated rodents given GGOH exhibited adverse changes in cardiac function. Vascular relaxation was also maintained following treatment with GGOH. The findings of this study demonstrate that GGOH can prevent statin-induced skeletal muscle fatigue in rodents without causing adverse changes in cardiovascular function. Further studies to elucidate the exact mechanisms underlying the effects observed in this investigation are warranted. (Translational Research 2019; 000:1–13)

Abbreviations: APA = action potential amplitude; APD = action potential duration; CO_2 = carbon dioxide; CON = control; CoQ10 = coenzyme Q10; CRC = concentration response curve; dF/dt = rate of change in force; +dP/dt = maximum rate of contraction; −dP/dt = maximum rate of relaxation; eNOS = endothelial nitric oxide synthase; FC = force of contraction; FFC = force-frequency curve; GGOH = geranylgeraniol; GGPP = geranylgeranyl pyrophosphate; GTPases = GTP-binding proteins; HDL = high density lipoprotein; KHB = Krebs-Henseleit buffer; LDL = low density lipoprotein; O_2 = oxygen; RMP = resting membrane potential; SIM = simvastatin; SAMS = statin-associated muscle symptoms; TPSS = Tyrode's physiological salt solution; TR90 = time to 90% relaxation

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Submitted for Publication May 24, 2019; received submitted August 1, 2019; accepted for publication August 13, 2019.

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1931-5244/$ - see front matter

https://doi.org/10.1016/j.trsl.2019.08.004

AT A GLANCE COMMENTARY

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Background

The depletion of geranylgeranyl pyrophosphate (GGPP) has been implicated in the pathogenesis of statin-associated muscle symptoms (SAMS). Accordingly, several in vitro studies have shown that the coadministration of GGPP (or its precursor, geranylgeraniol [GGOH]) with statins can improve the viability of skeletal muscle cells. The potential of GGPP repletion to prevent SAMS in vivo, however, has not been investigated.

Translational Significance

This investigation demonstrates that the administration of GGOH can prevent skeletal muscle fatigue in a rodent model of SAMS. This is the first evidence from an animal study to support that GGPP repletion is a feasible intervention for managing SAMS.

INTRODUCTION

Statins therapy is usually well-tolerated, but it can be associated with adverse side effects.[1] The most prevalent of these effects are statin-associated muscle symptoms (SAMS),[2] which range from mild muscle weakness/pain (ie, myalgia) to acute necrosis/rhabdomyolysis.[3,4] With the exception of rhabdomyolysis, it is generally held that the cardioprotective benefits conferred by statin therapy outweigh the discomfort caused by SAMS.[5,6] Nonetheless, the onset of these conditions is a significant contributor to statin noncompliance,[7] and thus, a considerable obstacle for improving cardiovascular outcomes.[8]

Currently in clinical practice, several strategies are employed to manage SAMS, including lower dose/alternate-day dosing,[9,10] vitamin D repletion,[11] and coenzyme Q10 (CoQ10) supplementation.[12] While these methods can be effective for alleviating SAMS, they do have limitations. For instance, not all individuals who take CoQ10 supplements experience a resolution of their symptoms.[13] Similarly, not all investigations have shown an association between serum vitamin D levels and the risk of statin-induced myotoxicity.[14-16] Furthermore, the adoption of less rigorous dosing protocols does not always guarantee the same degree of cardiovascular benefit compared to high-dose therapy.[17,18] Hence, more effective strategies for preventing statin-induced myotoxicity still need to be identified.

One such therapy may be to restore intramuscular geranylgeranyl pyrophosphate (GGPP) levels. GGPP is a product of the mevalonate pathway, and as such, its synthesis is inhibited by statins.[19-21] It is postulated that the relatively small pool of GGPP in skeletal muscle may be why this tissue is particularly sensitive to statin-induced toxicity.[22,23] GGPP is required for the synthesis of compounds such as CoQ10,[24] as well as the activation of several small GTP-binding proteins (GTPases), including the Rho GTPase family (eg, RhoA, Rac1, and Cdc42).[25] These proteins regulate a variety of processes including intracellular trafficking and signalling,[26,27] apoptosis,[28] skeletal myogenesis/differentiation,[25] and muscle contraction.[21-29] Several in vitro studies have demonstrated an association between GGPP depletion and statin-induced damage in skeletal myofibers/myocytes.[21,22,30-32] Moreover, these investigations have also shown that supplementation with GGPP (or its precursor, geranylgeraniol [GGOH]), can prevent this myotoxicity.

Nonetheless, the ability of GGPP/GGOH administration to alleviate SAMS in vivo remains to be investigated. Changes in RhoA activity in rodents with SAMS has been assessed,[33] however, the direct effects of GGPP repletion on statin-induced myotoxicity in animals is yet to be studied. The absence of this data may relate to concerns that GGPP/GGOH administration could negatively impact upon the cardioprotective effects of statins. Indeed, many of the pleiotropic effects associated with these medications, such as improved endothelial function and inhibition of cardiac fibrosis/hypertrophy, have been attributed to GGPP depletion and the consequent inactivation of small GTPases.[34-36]

Accordingly, the aim of this investigation was to conduct a feasibility study to determine whether administering GGOH could prevent skeletal muscle fatigue in a validated rodent model of SAMS. This model produces symptoms which are characteristic of statin-induced myalgia, namely a fiber-selective decline in force production in the absence of significant serum creatine kinase elevation.[37] In addition to changes in skeletal muscle function, alterations in myocardial and vascular performance were also assessed to establish the viability of GGOH/GGPP repletion for preventing SAMS.

MATERIALS AND METHODS

Ethical approval and animal treatment protocols. Female Wistar rats were sourced from the Central Queensland University Rodent Breeding colony (Rockhampton, Australia). Females typically demonstrate

greater sensitivity to the myotoxic effects of statins [38-41] and are thus appropriate for studying the pathogenesis of SAMS in rodent studies.[37] All of the treatments and protocols employed in this investigation were approved by the Institution's Animal Ethics Committee (Central Queensland University AEC: 20221). Rodents were housed at 22°C ± 2°C on a constant 12-hour light/dark cycle and allowed access to rat chow and water ad libitum. Once 12 weeks of age, the animals were randomized to 1 of 4 treatment groups: control (CON, $n = 23$), control with GGOH (CON + GGOH, $n = 23$), simvastatin (SIM, $n = 23$), or simvastatin with GGOH (SIM + GGOH, $n = 23$). Simvastatin was dissolved in a 10% v/v solution of polysorbate20 in milli-Q water and administered as a bolus dose of 80 mg kg^{-1} day^{-1}. GGPP was administered as its precursor, GGOH, owing to its greater cell/tissue permeability.[32] GGOH was dissolved in saline and delivered at a dose of 15 mg kg^{-1} day^{-1}. This concentration was used as it was calculated to be an equivalent mid-range dose (per kg bodyweight) to that which has previously been delivered to rats.[42] All interventions were administered via oral gavage for 14 days. In order to monitor the health of the animals during treatment, body mass and water/food consumption were assessed every 2 and 3 days, respectively. At the end of the dosing period, rodents were euthanized via a 1.0 mL intraperitoneal injection of sodium pentobarbitone (187.5 mg mL^{-1}).

Skeletal muscle organ baths. Skeletal muscle force-frequency curves were generated using electrical field stimulation, as previously described.[37] Gastrocnemius, soleus, and tibialis anterior muscles were promptly isolated from the hind limb. The muscles were positioned between 2 platinum zig-zag electrodes and submerged in warmed (37°C) 25 mL organ baths containing gassed (oxygen (O_2) 95%/carbon dioxide (CO_2) 5%) modified Krebs-Henseleit buffer (KHB, all in mM concentrations: sodium chloride 119, potassium chloride 5, magnesium sulfate 1, potassium dihydrogen phosphate 1, sodium hydrogen carbonate 25, glucose 11, and calcium chloride 2; pH ~ 7.4). The tissues were then stimulated at increasing frequencies between 1 and 100 Hz. Responses were measured using FT03 force transducers (Grass Technologies) and recorded using Lab Chart software (ADInstruments). The wet mass of isolated gastrocnemius, soleus, and tibialis anterior muscles at the time of euthanasia were also recorded.

Langendorff heart preparations. Left ventricular compliance was assessed using a modified version of the Langendorff heart preparation.[43] Intact hearts were rapidly excised and submerged in ice-cold modified KHB. The aortas were cleaned, cannulated, and then perfused with warmed (37°C), gassed (O_2 95%/CO_2 5%) modified KHB. A latex balloon was inserted into the left ventricle and the hearts were then paced at 250 beats per minute via electrical stimulation of the right atrium. Pressure-volume curves were generated by filling the balloon with milli-Q water at increasing increments between 0 mmHg and 30 mmHg. Using this data, the following parameters were assessed: diastolic stuffiness, maximum rate of contraction (+dP/dt), maximum rate of relaxation (−dP/dt), end systolic pressure, and developed pressure.

Single cell microelectrode studies. The electrophysiology of the myocardium was assessed as previously described.[44] Papillary muscles were dissected from the left ventricle and placed in a 1 mL chamber containing warmed (37°C), gassed (O_2 95%/CO_2 5%) Tyrode's physiological salt solution (TPSS, all in mM concentrations: sodium chloride 137, potassium chloride 5, magnesium chloride 1, sodium dihydrogen phosphate 0.4, sodium bicarbonate 23, calcium chloride 2, glucose 6, ascorbic acid 0.3, and ethylenediaminetetraacetic acid 0.1; pH ~ 7.4). Tissues were positioned between 2 platinum electrodes and attached to a modified SensoNor AE 801 micro-force transducer. Electrical field stimulation was used to induce contractions within the papillary muscle (1 Hz frequency, 0.5 ms pulse width, stimulus strength 20% above threshold). Tissues were then impaled with a glass electrode (filled with 3 M potassium chloride) and bioelectrical activity was recorded using a Cyto 721 electrometer (World Precision Instruments). Data pertaining to resting membrane potential, action potential amplitude, action potential duration at 20%, 50%, and 90% repolarisation (APD20, APD50, and APD90), force of contraction (FC), rate of change in force (dF/dt), and time to 90% relaxation (TR90) were collected. As the isolated hearts could not be used for both the Langendorff and microelectrode experiments, the sample size used for each experiment was 11−12 per group. The wet mass of the left ventricle was recorded upon the completion of each experiment.

Isolated blood vessel organ baths. Cleaned 5 mm sections of isolated thoracic aortas were threaded onto stainless steel hooks, anchored into organ baths containing TPSS, and allowed to equilibrate for 30 minutes.[45] Aortas were set to a resting tension of 10 mN and then cumulative concentration-response curves (CRCs) to acetylcholine, sodium nitroprusside, and noradrenaline were performed (bath concentration range: 1×10^{-9} M−3×10^{-4} M).

Second-order mesenteric arteries were dissected, cut into 2 mm segments and mounted into a wire myograph system (containing TPSS) using a 40-μm diameter stainless-steel wire (DMT-Asia Pacific).[45] Tissues were normalized to a transmural pressure of 100 mmHg and rested for 30 minutes. CRCs to acetylcholine, sodium

nitroprusside, and noradrenaline were performed. All CRCs were analyzed using nonlinear regression so that $Log_{10}EC50$ values could be calculated.

Quantitative reverse transcription PCR (RT-qPCR). mRNA was extracted from randomly selected muscle samples (n of 6 per group) using Trizolreagent (Applied Biosystems Inc.). cDNA was produced from mRNA using SuperscriptIII reverse transcriptase (Applied Biosystems Inc.), as per the manufacturer's instructions. Relative levels of target genes were analyzed using TaqmanMaster Mix and Gene Expression Assays (Applied Biosystems Inc.) on Rotor-Gene Q equipment (Qiagen). The results were normalized to *Gapdh* for calculation of relative gene expression.[46] The following genes of interest were investigated: Rn00591730_m1, *Atrogin-1* (muscle atrophy); Rn00590197_m1, *Murf-1* (muscle atrophy); Rn00585577_m1, *Pdk4* (carbohydrate metabolism); Rn00566193_m1, *Pparα* (lipid metabolism); Rn00690588_g1, *Sod2* (oxidative stress); Rn00821759_g1, *Mt1a* (oxidative stress); Rn01488777_g1, *Mhc-β* (cardiac hypertrophy); and Rn00580641_m1, *Bnp* (cardiac hypertrophy).

Serum lipid profiles. The lipid profiles of serum samples collected at the time of euthanasia (n of 8 per group) were assessed using a Roche Diagnostics Cobas Integra 400 plus analyzer.

Data and statistical analysis. All statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software). Data were evaluated for outliers (ROUT analysis) and normality (D'Agostino-Pearson test or Kolomogov-Smirnov test) prior to statistical testing. As all data was parametric, 1-way ANOVA, or repeated-measures 2-way ANOVA, with Tukey post-hoc tests (alpha < 0.05) was used to assess for statistically significant differences. The results are presented as means with standard deviations.

RESULTS

Skeletal muscle force production. Simple effects were analyzed for the force-frequency curves as all curves showed significant interaction. Gastrocnemius muscles isolated from the SIM rats exhibited significantly reduced force production in comparison to the CON group ($P < 0.05$) (Fig 1). This effect was completely abrogated by coadministration with GGOH ($P < 0.001$). The CON + GGOH rats also demonstrated greater function relative to the SIM animals from

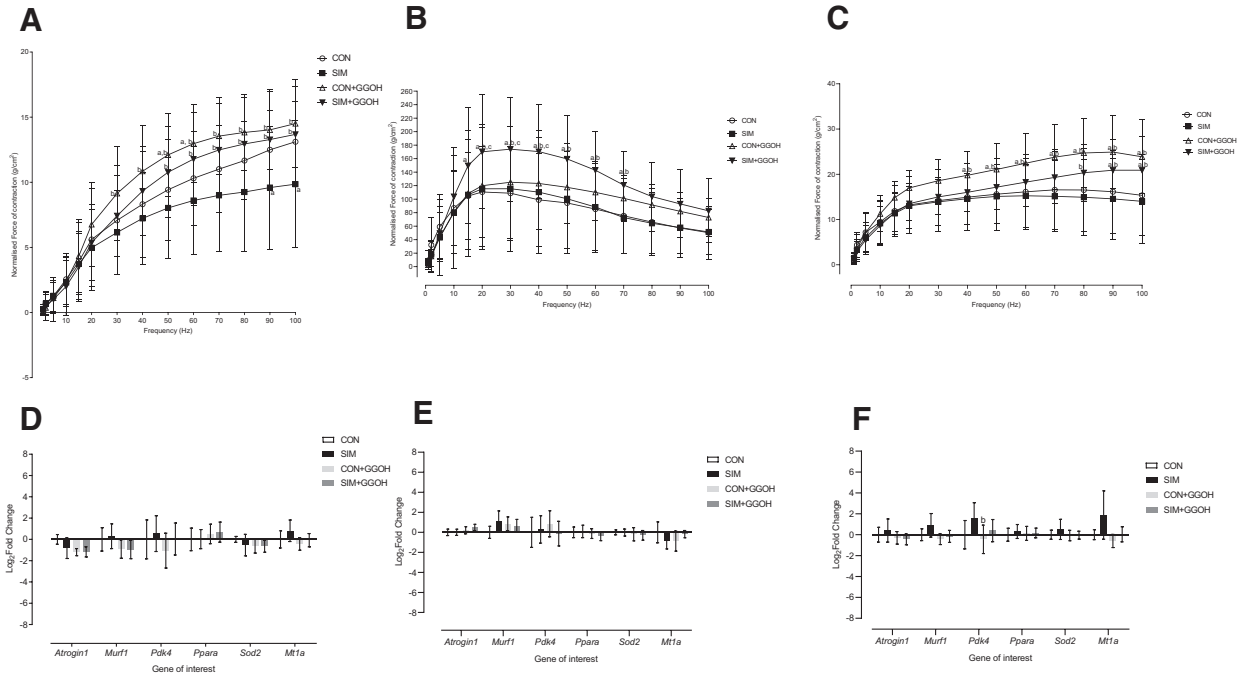

Fig 1. Normalised force-frequency response curves and mRNA expression in muscles isolated from control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats: (A) and (D) shows results for gastrocnemius muscles; (B) and (E) for soleus muscles and; (C) and (F) for tibialis anterior muscles. Results for the force-frequency curves are expressed as means with standard deviations (n of 16−23 per group following removal of inviable tissues and outliers by ROUT analysis). This data was analyzed using 2-way repeated measures ANOVA followed by Tukey posthoc test. Results for mRNA expression are expressed as mean Log_2 fold changes with standard deviations (n of 5−6 per group following removal of outliers by ROUT analysis). This data was analyzed using 1-way ANOVA followed by Tukey posthoc test. [a]Indicates $P < 0.05$ vs CON, [b]indicates $P < 0.05$ vs SIM, and [c]indicates $P < 0.05$ vs CON + GGOH.

30 Hz onward ($P < 0.001$). Correspondingly, maximum force production was significantly reduced in the SIM rats relative to the other treatment groups ($P < 0.05$) (Table I). Additionally, force production in the CON + GGOH group was significantly greater than the CON animals between 50 and 60 Hz ($P = 0.04$). These alterations in force production, however, occurred in the absence of any significant changes in muscle mass (Table I).

The soleus muscles isolated from the SIM and CON + GGOH animals exhibited comparable function to the CON group (Fig 1). Conversely, force production was significantly higher in the SIM + GGOH rats ($P < 0.05$). Specifically, the SIM + GGOH animals showed greater performance compared to the CON and SIM groups from 15 Hz and 20 Hz onward, respectively. Accordingly, maximum force was significantly increased in the SIM + GGOH animals relative to the CON and SIM rats ($P < 0.05$) (Table I). SIM + GGOH animals also showed greater responses compared to the CON + GGOH group between 20 and 40 Hz ($P < 0.05$), however maximum force production was not significantly different between these groups. There were no significant differences in soleus mass amongst the treatment groups.

The administration of GGOH also increased force production in the tibialis anterior (Fig 1). Specifically, the CON + GGOH animals exhibited significantly greater performance compared to the CON and SIM groups from 40 Hz onward ($P < 0.05$). Correspondingly, maximum force production was significantly greater in the CON + GGOH rats compared to the CON and SIM groups ($P = 0.002$ vs CON; $P = 0.005$ vs SIM). Force production in the tibialis anterior muscles isolated from the SIM + GGOH rats was also significantly higher relative to the CON and SIM animals from 90 Hz and 80 Hz onward, respectively ($P < 0.05$). However, 1-way ANOVA of maximum force production identified no statistically significant differences between the SIM + GGOH rats and the CON or SIM groups (possibly because of the relatively large within-group variability) (Table I). Tibialis anterior mass was similar across the 4 treatment groups (Table I).

Left ventricular performance and electrophysiology. Treatment with simvastatin and/or GGOH was not associated with any significant alterations in systolic function, diastolic function, or left ventricle mass (Table II). Single-cell microeletrode parameters, including resting membrane potential, action potential amplitude, APD, and FC, were also largely comparable amongst the treatment groups (Table II). The GGOH-treated animals did exhibit increased dF/dt values relative to the CON and SIM groups, but a statistically significant difference was only observed between the CON and CON + GGOH animals ($P = 0.03$). The SIM, CON + GGOH and SIM + GGOH rats all showed a reduction in TR90 compared to the CON group. Nonetheless, there was high within-group variability and a statistically significant difference was only apparent between the CON and SIM + GGOH animals ($P = 0.02$). APD90 did appear to be slightly prolonged in the statin-treated groups. The within-group values, however, were largely heterogeneous, thereby indicating the lack of a strong treatment-induced effect.

Lipid profiles. Total cholesterol, HDL cholesterol, and LDL cholesterol were not altered by treatment with simvastatin or GGOH alone. The SIM + GGOH animals, however, exhibited significantly greater LDL cholesterol levels in comparison to the other treatment groups ($P < 0.001$) (Table III). Serum triglycerides were significantly lower in the SIM, CON + GGOH, and SIM + GGOH rats in comparison to the CON group ($P < 0.05$).

Function of elastic arteries. Endothelium-dependent and -independent relaxation responses in isolated thoracic aortas were similar amongst the treatment groups (Fig 2). Nonetheless, the acetylcholine $Log_{10}EC50$ values were significantly reduced in the CON + GGOH and SIM + GGOH groups compared to the CON and SIM animals ($P < 0.05$) (Table IV). Conversely, the sodium nitroprusside $Log_{10}EC50$ values were significantly increased in the SIM, CON + GGOH, and SIM + GGOH groups relative to the CON rats ($P < 0.05$). Simple effects analysis of noradrenaline CRCs identified that both GGOH-treated groups exhibited significantly reduced contraction compared to the CON and SIM animals from 1×10^{-8} M to 3×10^{-7} M and 1×10^{-7} M to 3×10^{-7} M, respectively ($P < 0.05$). This effect, however, was absent at the higher doses of the CRC and consequently all groups exhibited similar maximum noradrenaline-induced contraction values. The average noradrenaline $Log_{10}EC50$ value for the CON + GGOH rats was significantly increased compared to the CON and SIM treatment groups ($P < 0.001$). $Log_{10}EC50$ was also higher in the SIM + GGOH rats relative to the CON and SIM animals, but this effect was only statistically significant in comparison to the SIM group ($P < 0.001$).

Function of muscular arteries. Simple effects were analyzed for the CRCs in the mesenteric arteries as all showed significant interaction. Mesenteric arteries from the GGOH groups demonstrated significant improvements in endothelium-dependent relaxation (Fig 2). Responses to acetylcholine were significantly greater in the CON + GGOH animals compared to the CON and SIM groups from 1×10^{-9} M to 1×10^{-8} M and 1×10^{-9} M to 3×10^{-7} M, respectively ($P < 0.05$). Similarly, the SIM + GGOH rats showed greater responses relative to the CON and SIM animals from 1×10^{-9} M to 3×10^{-8} M and 1×10^{-9} M to 1×10^{-6} M, respectively ($P < 0.05$). High intergroup variability, however, negated any statistical significance for the 1-way ANOVA of maximum endothelial-dependent relaxation (Table IV). The acetylcholine $Log_{10}EC50$ values were similar between the CON, SIM, and CON + GGOH groups, but the SIM + GGOH rats showed a significantly higher value compared to

Table I. Maximum force production and mass of skeletal muscles isolated from control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats

Parameter	CON	SIM	CON + GGOH	SIM + GGOH
Gastrocnemius maximum force (g/cm^2)	13.32 (3.35)	10.09 (4.56)*	14.85 (3.12)†	13.73 (3.68)†
Soleus maximum force (g/cm^2)	136.70 (61.81)	133.70 (88.18)	156.10 (70.16)	196.60 (70.84)*,†
Tibialis anterior maximum force (g/cm^2)	18.02 (9.01)	18.02 (7.56)	26.28 (8.04)*,†	21.74 (6.97)
Gastrocnemius mass (mg/g)	5.82 (0.37)	5.66 (0.51)	5.78 (0.34)	5.80 (0.33)
Soleus mass (mg/g)	0.51 (0.05)	0.51 (0.06)	0.52 (0.06)	0.53 (0.07)
Tibialis anterior mass (mg/g)	1.90 (0.19)	1.87 (0.13)	1.88 (0.13)	1.87 (0.12)

Results are expressed as means with standard deviations. *n* of 16–23 per group for maximum force following removal of inviable tissues and outliers by ROUT analysis. *n* of 22–23 per group for tissue mass following removal of outliers by ROUT analysis. Data was analyzed using 1-way ANOVA followed by Tukey posthoc test.
*Indicates $P < 0.05$ vs CON.
†Indicates $P < 0.05$ vs SIM.

Table II. Performance of left ventricles from control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats

Parameter	CON	SIM	CON + GGOH	SIM + GGOH
Langendorff parameters				
Diastolic stiffness (κ)	34.78 (7.21)	36.43 (4.86)	32.07 (5.15)	35.36 (4.87)
Max +dP/dt (mmHg/s)	2480.00 (473.70)	2354.00 (469.30)	2220.00 (250.60)	2259.00 (191.90)
Max −dP/dt (mmHg/s)	−1503.00 (362.50)	−1492.00 (287.20)	−1349.00 (180.20)	−1414.00 (141.70)
End systolic stiffness (mmHg)	109.60 (21.48)	109.80 (17.09)	108.00 (10.95)	109.90 (8.39)
Developed pressure (mmHg)	100.10 (21.55)	100.10 (17.82)	98.27 (11.02)	100.60 (8.00)
Electrophysiology parameters				
Resting membrane potential (mV)	−52.13 (21.13)	−57.97 (11.85)	−55.08 (12.15)	−59.03 (9.51)
Action potential amplitude (mV)	66.73 (19.04)	59.95 (16.95)	63.75 (11.43)	63.31 (11.47)
Action potential duration 20% (ms)	18.16 (5.37)	16.61 (4.87)	14.53 (2.91)	14.22 (2.57)
Action potential duration 50% (ms)	28.44 (8.19)	27.23 (9.96)	23.79 (3.82)	25.11 (3.96)
Action potential duration 90% (ms)	77.57 (25.69)	87.32 (35.89)	72.69 (12.10)	92.21 (19.12)
Force of contraction (mN)	0.81 (0.63)	1.06 (0.91)	1.59 (0.90)	1.52 (0.95)
dF/dt (V/s)	0.30 (0.21)	0.39 (0.34)	0.77 (0.52)*	0.74 (0.44)
TR90 (ms)	207.00 (78.89)	156.70 (69.11)	139.90 (7.44)	132.90 (21.11)†
Biometric parameters				
Left ventricle mass (mg/g)	2.37 (0.31)	2.51 (0.45)	2.26 (0.29)	2.42 (0.40)

Results are expressed as means with standard deviations. *n* of 11–12 per group for Langendorff parameters, *n* of 8–12 per group for electrophysiology parameters and *n* of 23 per group for biometric parameters following removal of inviable tissues and outliers by ROUT analysis. Data was analyzed using 1-way ANOVA followed by Tukey posthoc test.
*Indicates $P < 0.05$ vs CON.
†Indicates $P < 0.05$ vs SIM. dF/dt, rate of change in force; +dP/dt, maximum rate of contraction; −dP/dt, maximum rate of relaxation; TR90, time to 90% relaxation.

the other groups ($P < 0.05$). Endothelium-independent relaxation was similar amongst the treatment groups at the lower doses of the CRC. However, at the higher doses, the CON + GGOH, and SIM + GGOH groups exhibited significantly greater responses compared to the CON rats ($P < 0.05$). Accordingly, maximum endothelium-independent relaxation was significantly greater in the CON + GGOH and SIM + GGOH animals compared to the CON group ($P < 0.01$). Owing to the reduced responsiveness at the lower doses, the sodium nitroprusside $Log_{10}EC50$ values were significantly higher in the CON + GGOH and SIM + GGOH rats compared to the CON ($P < 0.05$) and SIM animals ($P < 0.001$). The GGOH-treated groups exhibited significantly reduced responsiveness to noradrenaline compared to the CON and SIM rats from 1×10^{-7} M and 1×10^{-6} M onward, respectively ($P < 0.05$). Correspondingly, maximum responses were significantly decreased in the CON + GGOH and SIM + GGOH groups relative to the CON and SIM rats ($P < 0.001$). The noradrenaline $Log_{10}EC50$ values were also significantly increased in the GGOH-treated groups ($P < 0.001$).

Relative gene expression. The expressions of *Atrogin-1*, *Murf-1*, *Pdk4*, *Pparα*, *Sod2*, and *Mt1a* in the gastrocnemius and soleus muscles were similar amongst the treatment groups (Fig 1). Similarly, isolated tibialis anterior muscles showed no changes in *Atrogin-1*, *Murf-1*, *Pparα*, or *Sod2* mRNA levels. *Pdk4* mRNA levels, however, were increased in the tibialis anterior muscles isolated from the SIM rats, but this difference was only just statistically significant in comparison to the CON + GGOH group ($P = 0.049$). There was a slight increase in the expression of *Mt1a* in the SIM80 rats; however, large within-group variability negated any statistically significant effect. Average *Bnp* expression was increased by 9-to-10-fold in the GGOH-treated groups, and by 2-fold in the SIM animals (Fig 3). Nonetheless, the within-group mRNA levels were largely heterogenous and no statistically significantly differences were observed. *Mhc-β* expression was also similar amongst the treatment groups. Additionally, preliminary assessment of serum cardiac biomarkers (including creatine kinase-MB and lactate dehydrogenase) showed no evidence of cardiac dysfunction following treatment with simvastatin and/or GGOH (data not shown).

Biometric indices of health status. As the 2-way ANOVAs of biometric evaluations showed significant interaction, simple effects were assessed. The SIM rats exhibited signs of physiological distress during the treatment period, as evidenced by significant reductions in water intake and food consumption ($P < 0.05$) (Fig 4).

Table III. Lipid profiles of control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats

Parameter	CON	SIM	CON+GGOH	SIM+GGOH
Total cholesterol (mmol/L)	1.90 (0.37)	1.81 (0.26)	1.88 (0.23)	2.14 (0.19)
HDL-C (mmol/L)	1.33 (0.21)	1.43 (0.32)	1.46 (0.21)	1.65 (0.23)
LDL-C (mmol/L)	0.13 (0.06)	0.18 (0.07)	0.19 (0.04)	0.35 (0.12)*,†,‡
Triglycerides (mmol/L)	1.18 (0.44)	0.57 (0.18)*	0.63 (0.28)*	0.42 (0.17)*

Results are expressed as means with standard deviations. *n* of 6–8 per group following removal of inviable tissues and outliers by ROUT analysis. Data was analyzed using 1-way ANOVA followed by Tukey posthoc test.
*Indicates $P < 0.05$ vs CON.
†Indicates $P < 0.05$ vs SIM.
‡Indicates $P < 0.05$ vs CON + GGOH. HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

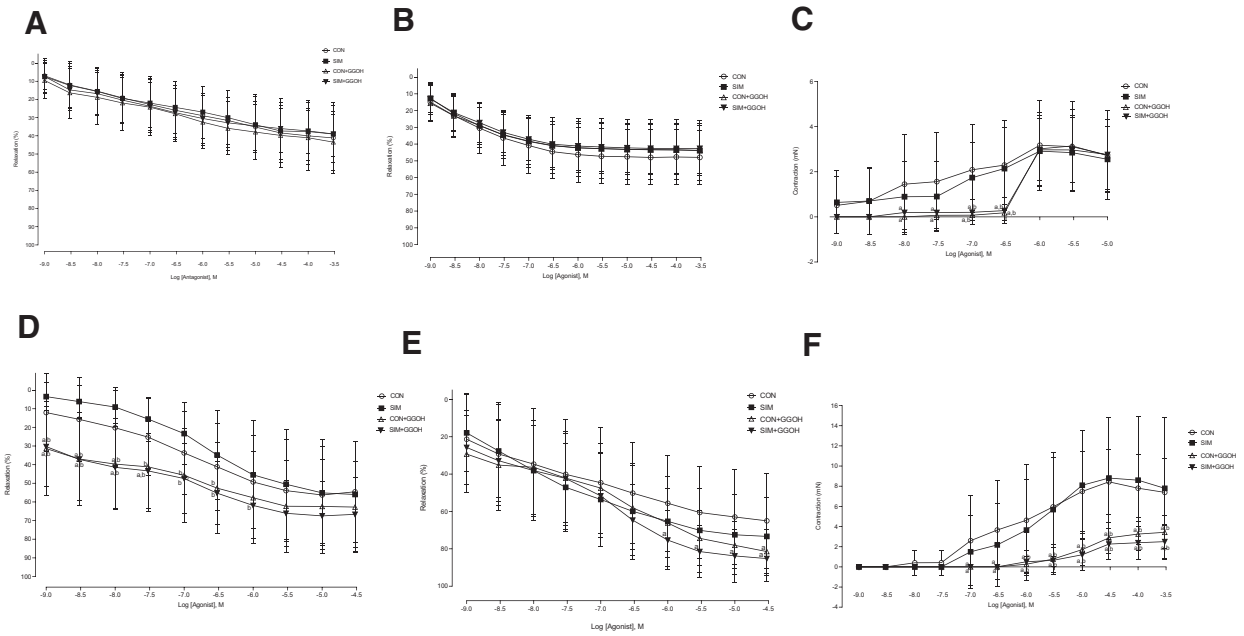

Fig 2. Concentration response curves in thoracic aortas (A−C) and mesenteric arteries (D−F) in muscles isolated from control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats: (A) and (D) shows results for acetylcholine; (B) and (E) for sodium nitroprusside and; (C) and (F) for noradrenaline. Results are expressed as means with standard deviations (n of 21−23 or 12−19 per group for aortas and mesenteric arteries, respectively, following removal of inviable tissues and outliers by ROUT analysis). Data was analyzed using 2-way repeated measures ANOVA followed by Tukey posthoc test. Relaxation responses were normalized to percentage relaxation of maximum noradrenaline-induced precontraction. [a]Indicates $P < 0.05$ versus CON and [b]indicates $P < 0.05$ vs SIM.

Table IV. $Log_{10}EC50$ and maximum responses in thoracic aortas and mesenteric arteries isolated from control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats

Parameter	CON	SIM	CON+GGOH	SIM+GGOH
Aorta acetylcholine $Log_{10}EC50$	−6.71 (1.05)	−6.78 (1.05)	−6.98 (1.24)*,†	−7.03 (1.08)*,†
Aorta sodium nitroprusside $Log_{10}EC50$	−7.82 (0.74)	−7.99 (0.56)*	−7.90 (0.51)*	−7.94 (0.47)*
Aorta noradrenaline $Log_{10}EC50$	−6.79 (2.11)	−6.75 (1.88)	−6.25 (0.41)*,†	−6.23 (0.57)*,†
Aorta acetylcholine maximum relaxation (% relaxation)	41.62 (19.37)	39.49 (15.25)	43.94 (15.48)	39.37 (12.38)
Aorta sodium nitroprusside maximum relaxation (% relaxation)	48.89 (15.93)	44.29 (14.96)	44.05 (17.70)	43.15 (14.80)
Aorta noradrenaline maximum contraction (mN)	3.81 (2.04)	3.48 (1.46)	3.31 (1.69)	3.44 (1.31)
Mesenteric artery acetylcholine $Log_{10}EC50$	−6.88 (1.10)	−6.72 (0.77)	−6.87 (1.22)	−7.17 (1.30)*,†,‡
Mesenteric artery sodium nitroprusside $Log_{10}EC50$	−7.27 (1.17)	−7.44 (0.85)	−6.96 (1.14)*,†	−6.94 (0.75)*,†
Mesenteric artery noradrenaline $Log_{10}EC50$	−5.87 (1.37)	−5.82 (1.29)	−4.96 (0.75)*,†	−4.87 (0.60)*,†
Mesenteric artery acetylcholine maximum relaxation (% relaxation)	60.18 (27.65)	57.75 (28.04)	65.54 (23.22)	71.12 (15.50)
Mesenteric artery sodium nitroprusside maximum relaxation (% relaxation)	65.58 (24.64)	73.73 (20.93)	81.43 (11.69)*	85.69 (12.22)*
Mesenteric artery noradrenaline maximum contraction (mN)	9.23 (3.38)	9.72 (6.28)	3.76 (1.58)*,†	2.66 (1.67)*,†

Results are expressed as means with standard deviations. n of 21−23 per group for aortas following removal of inviable tissues and outliers by ROUT analysis. n of 12−19 per group for mesenteric arteries following removal of outliers by ROUT analysis. Data was analyzed using 1-way ANOVA followed by Tukey posthoc test.
*Indicates $P < 0.05$ vs CON.
†Indicates $P < 0.05$ vs SIM.
‡Indicates $P < 0.05$ vs CON+GGOH.

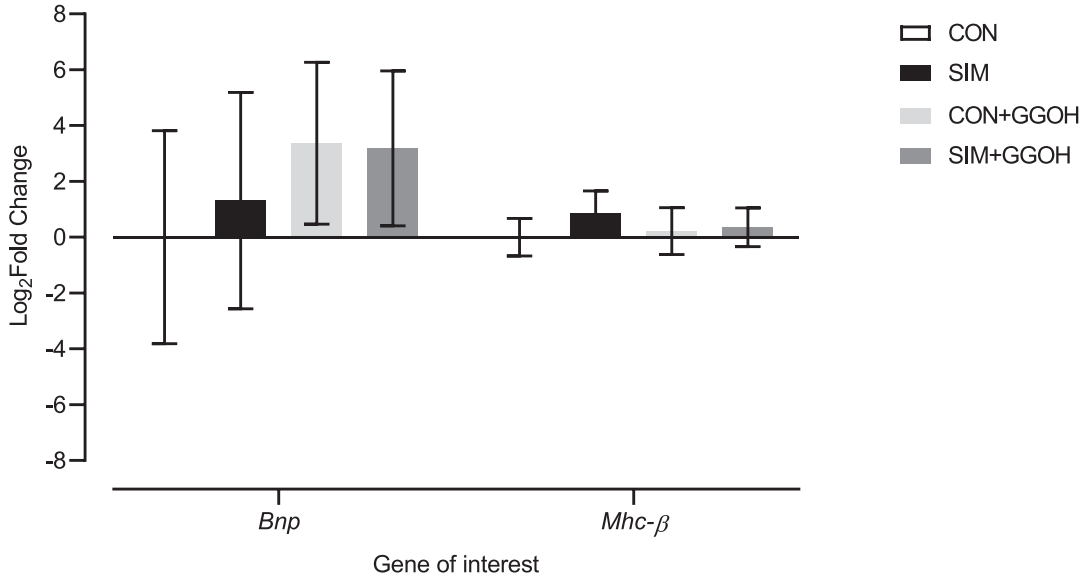

Fig 3. mRNA expression in left ventricles isolated from control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats. Results are expressed as mean Log_2 fold changes with standard deviations (n of 6 per group). Data was analyzed using 1-way ANOVA followed by Tukey posthoc test.

A reduction in the ability to gain body mass was also observed in the SIM group ($P < 0.05$) (Fig 4). The SIM + GGOH animals exhibited decreased water and food intake relative to the CON and CON + GGOH groups until day 7 of treatment ($P < 0.05$), after which consumption improved to control levels. GGOH did not increase weight gain in the statin-treated animals. The CON + GGOH rats maintained comparable water intake, food consumption, and body mass to the CON group for the whole of the treatment period.

DISCUSSION

This investigation has verified findings from in vitro studies[22,27,30,32] and demonstrated that supplementation with GGOH can prevent statin-induced skeletal muscle fatigue in rodents. Gastrocnemius muscles isolated from the SIM group exhibited a significant decline in muscle force production, an effect which is characteristic of this rodent model as well as statin-induced myalgia in clinical practice.[37] Treatment with GGOH completely abrogated skeletal muscle fatigue the gastrocnemius. The administration of this compound also alleviated other signs of physiological distress in the SIM animals including reduced food and water consumption. Both SIM-treated groups exhibited a reduction in body weight gain compared to the control animals. As the CON + GGOH rats did not exhibit this feature, the reduced body mass in the SIM + GGOH group was likely due to statin treatment. We have previously observed that simvastatin can cause weight loss in the absence of myalgia.[37] Furthermore, reduced weight gain has been reported in high-fat diet-fed rats given fluvastatin, and this was also not concluded to be an adverse effect.[47] Accordingly, the lower body mass observed in the SIM-treated groups in the present study is not considered to be pathological.

As this was a feasibility study, a comprehensive analysis of the molecular mechanisms responsible for the observed functional changes was not conducted. Nonetheless, preliminary assessment of these factors suggested that muscle atrophy (including changes in *Atrogin-1* and *Murf-1* expression) is not central to the development of statin-induced muscle fatigue. While this finding contrasts with other reports,[48,49] it supports our prior observations that significant muscle atrophy does not occur in this rodent model of SAMS (unpublished results). Impaired skeletal muscle metabolism has also been implicated in the pathogenesis of statin-induced myotoxicity.[21-23,33,50] However, the functional changes observed in isolated gastrocnemius muscles in this study were not associated with alterations in *Pdk4* or *Pparα* expression. Additionally, the mRNA levels of genes related to oxidative stress (ie, *Sod2* and *Mt1a*) did not correlate with muscle performance. Although these findings need to be validated by further mechanistic studies, they suggest that other cellular factors may play a more pivotal role in the onset of myalgia.

Contrary to the gastrocnemius, the soleus was not significantly impaired following treatment with simvastatin. This observation reflects that this muscle is predominately comprised of slow-oxidative muscle fibers, thereby making it largely resistant to statin-induced

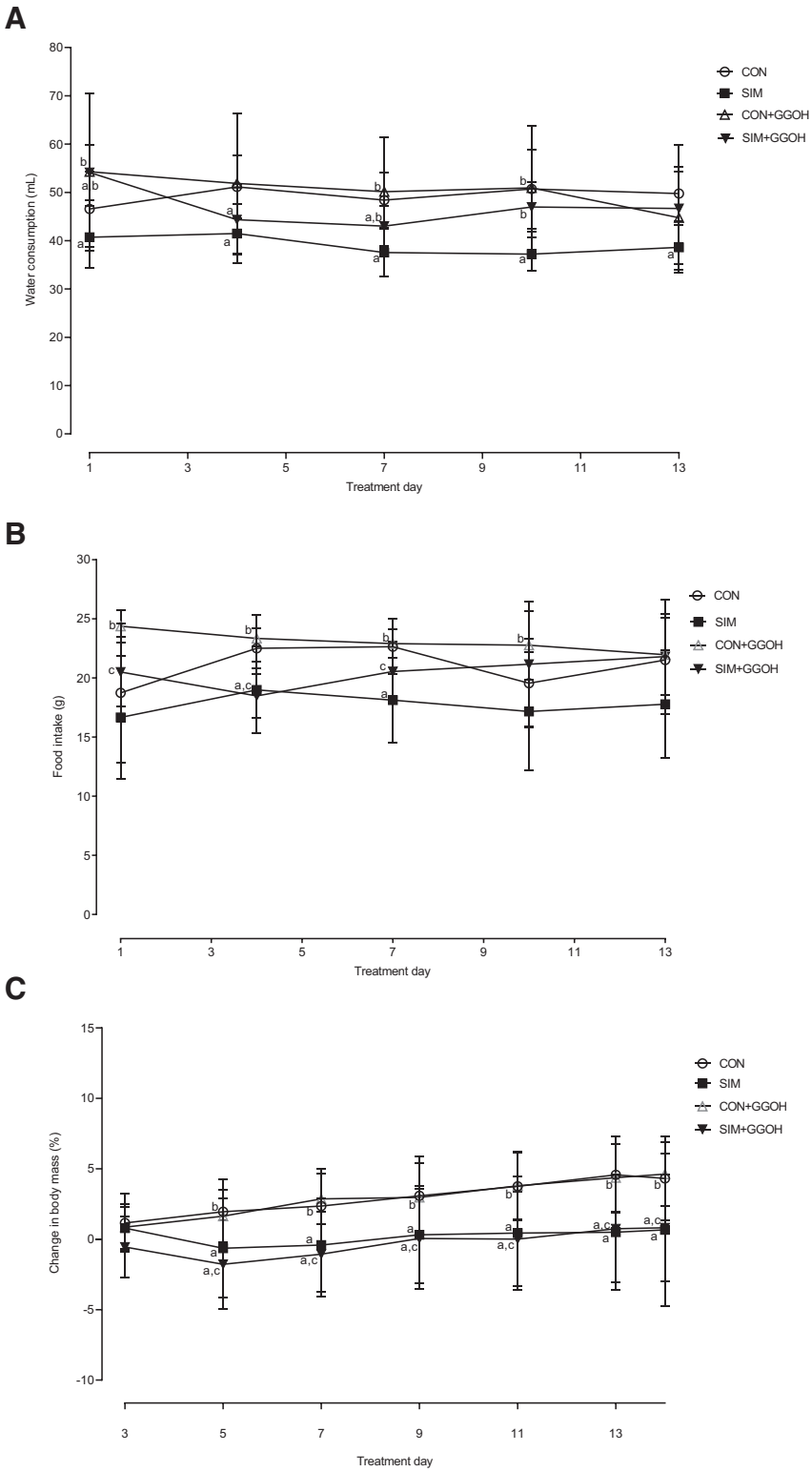

Fig 4. (A) Water consumption, (B) food intake, and (C) percentage change in body mass during treatment for control (CON and CON + GGOH) and statin-treated (SIM and SIM + GGOH) rats. Percentage change in body mass is normalized to day 1 of treatment. Results are expressed as means with standard deviations (n of 23 per group). Data was analyzed using 2-way repeated measures ANOVA followed by Tukey posthoc test. [a]Indicates $P < 0.05$ vs CON, [b]indicates $P < 0.05$ vs SIM, and [c]indicates $P < 0.05$ vs CON + GGOH.

myotoxicity.[51-53] Interestingly, the SIM + GGOH rats exhibited significantly increased force production in the soleus compared to the other treatment groups. Akin to the gastrocnemius, this effect occurred without any alterations in the mRNA expression profile. It is unclear why only the SIM + GGOH rats, and not the CON + GGOH animals, exhibited this improvement in muscle function. It is possible that a combination of increased intracellular calcium due to statin treatment,[54] and heighted calcium sensitivity of myosin due to increased RhoA activity,[55] facilitated the improvement in muscle contraction. Nonetheless, without directly assessing these parameters, the definitive cause of this effect is unclear.

Treatment with GGOH significantly improved the performance of the tibialis anterior, though in this case the effect was evident in both the CON + GGOH and SIM + GGOH groups. A statistically significant difference in *Pdk4* expression was observed between the CON + GGOH and SIM animals, however, this change did not correlate with the alterations in muscle function. For instance, the CON + GGOH rats exhibited greater force production relative to the CON animals, yet *Pdk4* mRNA levels was comparable between these 2 groups. Hence, further investigations are required to establish the cellular mechanism/s underlying the functional changes induced by GGOH.

For GGOH/GGPP repletion to be considered a viable treatment for SAMS, it must be able to improve skeletal muscle performance without causing any adverse changes in cardiovascular physiology. In this study, treatment with GGOH did not impair left ventricular performance in young, healthy rats. The absence of increased left ventricular mass or *Mhc-β* expression indicated that hypertrophy has not occurred following treatment with this compound. This finding is significant as increased Rho/Rac activity has been implicated in the pathogenesis of cardiac hypertrophy.[56] The mRNA levels of *Bnp* did appear to be increased in the CON + GGOH and SIM + GGOH rats. Nonetheless, the expression of this gene was largely heterogeneous which, in the absence of significant changes in contractile function, indicates that this was not a noteworthy treatment-induced effect.

In terms of left ventricular electrophysiology, treatment with GGOH was associated with a reduction in TR90. As there was no shortening of APD, however, the exact biological significance of this change is unclear. The GGOH-treated groups also exhibited an increase in dF/dt, possibly because of a slight increase in FC. Prolonged TR90 is reported to be associated with contractile dysfunction.[57,58] Similarly, reduced dF/dt in cardiovascular disease can be indicative of impaired myocardial contractility.[59] Hence, these findings suggest that the administration of GGOH did not have a detrimental effect on left ventricular electrophysiology.

There was no evidence of impaired endothelial function in either the CON + GGOH or SIM + GGOH groups. Instead, these animals exhibited improved endothelium-dependent relaxation, particularly in the mesenteric arteries. This effect may be the result of enhanced nitric oxide production mediated by increased Rac1 activity.[60] A direct assessment of changes in GTPase activity is required in order to validate this postulation. The acetylcholine $Log_{10}EC50$ values were significantly increased in the aortas isolated from the GGOH-treated groups. Typically, this change would imply a reduced sensitivity to acetylcholine.[61] However, as this result was not accompanied by impaired relaxation, it appears to have been of negligible physiological significance in this instance. Furthermore, the mesenteric arteries isolated from the CON + GGOH rats exhibited similar relaxation to the SIM + GGOH animals, yet the acetylcholine $Log_{10}EC50$ values differed significantly between these groups. Ultimately, these results suggest that altered endothelial performance was not solely responsible for the observed changes in endothelium-dependent relaxation following treatment with GGOH.

Endothelium-independent relaxation in the aortas was similar across the 4 treatment groups. Again, however, GGOH administration improved relaxation responses in the mesenteric arteries. The $Log_{10}EC50$ values for sodium nitroprusside were also significantly reduced in the CON + GGOH and SIM + GGOH groups. This finding suggests that the sensitivity of the vascular smooth muscle to nitric oxide had been increased by GGOH.[62] Rac1 activity is known to increase cGMP levels in vascular smooth muscle cells, and thereby enhance vasorelaxation.[59] Hence, it is possible that the observed improvements in endothelial-independent relaxation may have been mediated by an increase in Rac1 activity.

In terms of contractile responses, the CON + GGOH and SIM + GGOH animals exhibited decreased vascular sensitivity to noradrenaline, particularly in the mesenteric arteries. Reductions in noradrenaline-induced contraction can be associated with altered myofilament calcium sensitivity.[63] As both Rho and Rac1 are postulated to increase calcium sensitisation in vascular smooth muscle,[64-66] the loss of contractile response in these animals was unexpected. These results may reflect a differential effect of GTPases on vascular smooth muscle function under normal vs pathological conditions. Accordingly, further investigations are required to establish the mechanisms which may underlie this variability.

Treatment with simvastatin did not cause a significant reduction in total or LDL cholesterol levels. This finding was not unexpected as the cholesterol-lowering effect of statins is frequently absent in studies using normocholesterolemic rats.[67,68] Supplementation with GGOH alone also had no significant effect on total or LDL cholesterol concentrations. The SIM + GGOH animals, however, exhibited significantly greater LDL cholesterol levels compared to the other treatment groups. As this outcome was not observed in the CON + GGOH rats, it may indicate an antagonistic effect of GGOH and simvastatin on LDL cholesterol. Conversely, the change may have been due to a compensatory rise in hepatic cholesterol production in response to statin treatment,[69] or potentially an increase in squalene synthase activity due to increased GGOH/GGPP (ie, reduced competition between GGPP synthase and squalene synthase for farnesyl diphosphate).[70] The underlying mechanism needs to be verified as it has significant implications for the viability of GGOH/GGPP repletion as a treatment of SAMS.

Despite the anomalous effects on LDL cholesterol, the SIM + GGOH rats exhibited lower serum triglyceride levels in comparison to the CON group, as did the SIM and CON + GGOH rats. Little is known about the role of geranylgeranylated proteins in regulating serum triglyceride levels. Consequently, the precise cause for the reduction in triglyceride levels in the GGOH-treated animals is unclear. Statins are postulated to reduce serum triglyceride levels by increasing lipoprotein lipase activity[71] as well as the secretion of VLDL.[72] Accordingly, these effects may account for the decreased level of triglycerides in the SIM and SIM + GGOH rats. Moreover, as the latter demonstrated slightly lower concentrations compared to the SIM and CON + GGOH groups, it is possible that the coadministration of simvastatin and GGOH may exert a synergistic effect on triglyceride reduction.

A noteworthy limitation of this work is that the protein levels of the genes assessed in this investigation were not evaluated. As this was a point-of-concept study, it was outside the scope of this work to rigorously evaluate the molecular factors responsible for the observed functional responses. Accordingly, the preliminary findings generated by this study need to be validated by other investigations. Similarly, the exact alterations in GTPase activities (particularly RhoA and Rac1) which are induced following treatment with statins and/or GGOH need to be determined. It is also pertinent to establish whether the combined effects of GGOH and statin administration on cardiac and vascular smooth muscle differ in the presence of cardiovascular disease.

In conclusion, this study is amongst the first to demonstrate that the administration of GGOH can prevent skeletal muscle fatigue in a rodent model of statin-induced myalgia. Furthermore, GGOH elicited these effects without causing any adverse alterations in myocardial contractility or vasorelaxation. Hence, these results suggest that GGOH/GGPP repletion may be a viable option for managing SAMS. Nonetheless, there was evidence of impaired vasoconstriction in the GGOH-treated animals, as well as an increase in LDL cholesterol levels when coadministered with simvastatin. Further investigations are thus needed in order to validate the suitability of GGOH/GGPP for managing SAMS.

ACKNOWLEDGMENTS

Conflicts of Interest: All authors have read the Journal's policy on conflicts of interest and declare no potential conflicts of interest. The authors have read the Journal's authorship agreement, approved the final version of the manuscript, and agree to be accountable for all aspects of the work. All persons involved in this work who qualify for authorship are listed as authors.

This project was supported by the Australian Government Research Training Scheme and Research Training Scheme Stipend. The funding source was not involved in any of the following: study design; collection, analysis, and interpretation of data; writing of the report; or decision to submit the article for publication.

The authors would like to express their gratitude to Gina Richter-Stretton and Kimberly Ryan for their time and assistance with the laboratory experiments.

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CHAPTER 7

Extended Discussion

Preamble

This chapter contextualises the findings of this work in terms of the existing scientific literature on this topic. The implications of the major outcomes generated from this project are discussed, as well as future research directions which will expound the results of this investigation.

The Vancouver style of referencing has been used in this chapter owing to its preference as a referencing style in the Medical Sciences.

7. Extended Discussion

7.1. Overview of major findings

This research project aimed to address the following objectives: (i) clarify the influence of statin dose and lipophilicity on the pathogenesis of SAMS; (ii) assess the viability of GGPP administration (in the form of GGOH) to prevent SAMS *in vivo* and; (iii) evaluate cardiac and vascular smooth muscle performance in the presence of SAMS. By addressing these objectives, several novel and important findings have been generated from this project. These major outcomes can be summarised as follows:

- Major outcome 1: The genetic changes associated with statin-induced myotoxicity vary between different forms of SAMS;
- Major outcome 2: Functional assessments of skeletal muscle performance are important for assessing the physiological significance of molecular/biochemical changes in statin-induced myalgia;
- Major outcome 3: Lipophilicity and dose can significantly influence the myotoxic potential of statins;
- Major outcome 4: The development of statin-induced myalgia is not associated with adverse changes in cardiac or vascular smooth muscle, but the effects of statins on these parameters could vary with lipophilicity;
- Major outcome 5: GGPP supplementation (in the form of GGOH) can abrogate statin-induced muscle fatigue *in vivo*;
- Major outcome 6: Validating rodent models of SAMS can significantly reduce inter-study variability, as well as ambiguity surrounding the pathogenesis of these conditions.

This chapter will elucidate the significance of these outcomes, as well as their implications for future research on this topic.

7.2. Major outcome 1: *The genetic changes associated with statin-induced myotoxicity vary between different forms of SAMS*

7.2.1. Summary

Recent evidence indicates that while the clinical variants of SAMS are related, they are also distinct entities with varying aetiologies [1, 2]. The similarity/differences between the mechanisms underlying the different forms of SAMS, however, have not been thoroughly investigated. This project has provided quantitative evidence to support that the molecular alterations which occur during mild forms of SAMS (namely statin-induced myalgia) differ from the more severe manifestations. Specifically, changes (or lack thereof) in the expression of genes related to muscle atrophy, oxidative stress, myosin heavy chain fiber type composition, mitochondrial biogenesis and metabolism are not correlated with statin-induced muscle fatigue. Indeed, the SIM80 animals studied in this project exhibited significantly reduced force production (particularly in the gastrocnemius), but shared similar skeletal muscle mRNA expression profiles with the CON, SIM40, PRAV160 and SIM + GGOH groups.

7.2.2. Significance

This finding has important implications for developing more effective strategies to manage SAMS. Currently, the treatment of SAMS is stratified according to the severity of symptoms

being experienced. Statin therapy is either continued if muscle symptoms are tolerable (albeit using a lower dose/alternate-day dosing), or ceased in cases of rhabdomyolysis/myonecrosis [3, 4]. While effective, this approach is not desirable in terms of improving cardiovascular outcomes [5, 6]. Treatments which can be co-administered with statins to prevent myotoxicity are being trialled (e.g. coenzyme Q10 and vitamin D); however, as mentioned in Chapter 5, these interventions have had varied success [7, 8]. The results of the present investigation suggest that this inefficiency may relate to variation in the suitability of these interventions for treating particular forms of SAMS. For example, interventions aiming to treat mitochondrial toxicity caused by statins (such as coenzyme Q10) are likely only to be effective in cases where mitochondrial dysfunction is central to the development of SAMS. In order to identify more effective interventions for managing statin-induced myotoxicity, the precise mechanisms underlying each manifestation of SAMS need to be elucidated [1, 9]. The findings of this project build upon this notion and demonstrate that these therapies are likely to differ for each form of SAMS due to variation in the key factors causing these conditions.

The data generated by this investigation also has important implications for future research aiming to improve the accuracy of diagnosing SAMS in the clinical setting. The discrepancy between the frequency of statin-induced myotoxicity reported in RCTs and observational studies has placed increasing emphasis on the prevalence of the nocebo effect in clinical practice [10, 11]. Although some cases of SAMS are indeed attributable to the nocebo effect, the results of this project highlight that the absence of significant CK elevation/muscle atrophy/mitochondrial dysfunction does not rule-out the presence of statin-induced muscle myalgia. This work demonstrates that statins can cause significant muscle weakness/fatigue without gross changes in muscle structure. Accordingly, more accurate methods for

diagnosing SAMS are required as this will enable mild variants to be better distinguished from the nocebo effect, and vice versa (see discussion in section 7.3.). In turn, achieving this outcome will have important implications for improving quality of life, and cardiovascular outcomes, in statin users experiencing muscle discomfort.

7.2.3. Future directions

As mentioned above, the mechanisms underlying each type of SAMS need to be fully expounded so that the most appropriate interventions for treating each variant can be identified. The rodent model of statin-induced myalgia used in this project was effective for clarifying some key features of this form of SAMS (see section 7.7. for further discussion). Nonetheless, further work is required to elucidate the exact mechanisms underlying the physiological effects observed in this project (see section 7.8.3. for further discussion). It is recommended that these factors are evaluated using the SIM80 model in order to reduce any variability/ambiguity between the studies on this topic.

7.3. Major outcome 2: *Functional assessments of skeletal muscle performance are important for assessing the physiological significance of molecular/biochemical changes in statin-induced myalgia*

7.3.1. Summary

Skeletal muscle performance is not always measured in mechanistic studies of SAMS. The findings of this project, however, have identified that functional assessments are required in order to evaluate the physiological significance of molecular and/or biochemical changes in

skeletal muscle. This point is particularly relevant for the latter as biomarkers of muscle damage are reported to be unreliable for monitoring SAMS [12, 13]. Indeed, in the present project, SIM40 animals demonstrated significant elevations in serum myoglobin, H-FABP and CK-MM levels, but there was no evidence of impaired skeletal muscle function in these rats. Similarly, as described in section 7.2., gene expression profiles were not closely associated with changes in muscle performance.

7.3.2. Significance

This outcome has significant implications for the design of future mechanistic studies investigating SAMS. Specifically, the results assert that such investigations should include functional assessments of skeletal muscle performance. This data is especially required in studies investigating mild forms of SAMS as significant biochemical markers of damage can be absent in these conditions [14]. Assessing skeletal muscle function will also clarify the physiological significance of molecular/cellular changes which may be observed in certain types of statin-induced myotoxicity. Hence, this evaluation can reduce variability and ambiguity concerning the exact mechanisms underlying these conditions. Furthermore, as fatigue is a key indicator of skeletal muscle damage [15, 16], the inclusion of functional analyses can facilitate a more robust assessment of skeletal muscle integrity.

Evaluating skeletal muscle function will also enable more accurate differentiation between the various manifestations of SAMS. Historically, serum CK levels have been used for monitoring/diagnosing statin-induced myotoxicity [17]. It is increasingly apparent, however, that assessing serum CK alone is not sufficient for distinguishing between the different forms of SAMS [12, 13]. Furthermore, as a consequence of its unreliability, expert panels do not

recommend monitoring CK levels during statin treatment [18, 19]. Assessing changes in skeletal muscle performance can thus provide an alternative avenue for diagnosing SAMS. Furthermore, as not all forms of statin-induced myotoxicity are associated with muscle weakness/fatigue [18]; measuring changes in muscle function can assist in differentiating one type of SAMS from another.

Additionally, employing function-based criteria for diagnosing SAMS may also facilitate more accurate segregation of the nocebo effect from actual cases of SAMS (see also discussion in section 7.2.). As CK elevation is absent in mild forms of SAMS, detecting these variants currently relies on verbal complaints of skeletal muscle discomfort [20]. Such information, however, is subjective and may confuse "true" instances of myotoxicity with the nocebo effect [21]. Alternatively, using standardised assessments of skeletal muscle performance – such as the six minute walk test, repeated chair stands assessment, gait speed measurement, stair test, grip strength evaluation, etc. – would provide quantitative evidence to either confirm or refute patient complaints.

7.3.3. Future directions

The findings of this study reiterate the importance of developing more effective methods for diagnosing statin-induced myotoxicity. This fact is particularly pertinent as there is currently no standardised "gold standard" criteria for detecting or monitoring SAMS [18]. The results of this study suggest that any new method/criteria for diagnosing SAMS should include quantitative assessments of skeletal muscle performance. Additionally, it is recommended that future mechanistic studies of SAMS measure changes in skeletal muscle function so that the biological relevance of any cellular/biochemical anomalies can be evaluated. In turn,

these assessments will allow for more effective and targeted treatments for SAMS to be developed.

7.4. Major outcome 3: *Lipophilicity and dose can significantly influence the myotoxic potential of statins*

7.4.1. Summary

This project has demonstrated that the myotoxic effects of statins can be influenced by lipophilicity and dose. This point is exemplified by that fact that only a high-dose treatment regimen (i.e. 80 mg kg^{-1} day^{-1}) using a lipophilic statin (i.e. simvastatin) was able to induce changes in skeletal muscle integrity which were characteristic of SAMS. For instance, even when delivered at an equipotent dose to simvastatin, pravastatin showed no evidence of impairing skeletal muscle integrity. Similarly, the SIM50 treatment protocol did not induce adverse changes in gastrocnemius function, despite the dosing period being approximately twice as long as that of the SIM80 model. Furthermore, the results presented in Chapter 4 demonstrated a clear dose-response effect of statin treatment on skeletal muscle performance. In this regard, the findings of this project also reiterate the discrepancy between RCTs and observational/preclinical studies in terms of the prevalence of SAMS, as well as the factors influencing its development. Indeed, in contrast to the rodent-based investigations, the meta-analysis presented in Chapter 2 showed little evidence that the risk of SAMS was impacted upon by lipophilicity or dose.

7.4.2. Significance

While the results from the rodent-based investigations differed from those of the meta-analysis, paradoxically, this variation may actually provide some clarification regarding the role of pharmacological factors in the pathogenesis of SAMS. Specifically, although the rodent-based studies validated that lipophilicity and dose can affect the myotoxic potential of statins, the meta-analysis suggests that this impact is altered in the presence of other factors (e.g. polypharmacy, history of statin-intolerance, etc.). It is important to note that the rats used in this study were largely resistant to statin-induced myotoxicity, akin to most individuals prescribed statins [22]. Accordingly, the results of the rodent-based studies indicate that, in the absence of other risk factors for SAMS, pharmacological factors can be key determinants of statin-induced muscle damage. Conversely, in statin-intolerant individuals/persons with risk other factors for SAMS, lipophilicity and dose may not have as great of influence on the risk of myotoxicity. Specifically, statin-induced damage may occur when using low-dose treatment regimens and/or hydrophilic statins, as in the case of persons with statin-intolerance [23]. Hence, the findings of this project are important as they provide information which can help to explain this observation from clinical practice.

There has been controversy surrounding the ability of pravastatin to enter the skeletal muscle and exert significant physiological effects [24]. The results of this project are thus significant as they demonstrate that pravastatin can indeed affect skeletal muscle physiology. Although pravastatin exerted a beneficial effect in the present investigation, the findings provide some context for reports of pravastatin-induced myotoxicity in clinical practice [25, 26]. Further investigation into the factors which influence whether pravastatin produces detrimental or positive changes in skeletal muscle physiology will assist in clarifying the mechanisms underlying SAMS.

On this note, the findings of this project are also significant as they have provided quantitative evidence that statins can exert beneficial effects in skeletal muscle. This outcome was observed not only in those rodents given high-dose pravastatin, but also the animals treated with low-dose simvastatin. There are instances of statin-users exhibiting greater muscle performance compared to non-statin users [27]. Similarly, these medications have also been shown to induce beneficial changes in rodents with muscular dystrophy [28]. Nonetheless, few studies have investigated the mechanisms by which statins may be able to improve skeletal muscle performance. While this project has identified that changes in genes related to atrophy/metabolism/oxidative stress are not associated with statin-induced muscle fatigue, further investigations are needed to identify the processes underlying the observed improvements in muscle function (see section 7.8.3. for further discussion). In turn, comparing the alterations in these processes to their status during statin-induced myotoxicity will improve current understanding of how statins affect skeletal muscle physiology.

7.4.3. Future directions

As noted above, the factors which underlie the differential effects of statins on skeletal muscle require further investigation. Comparing these processes will assist in elucidating the pathogenesis of SAMS, as well as those factors which may increase the risk of SAMS in certain individuals. In turn, this information will also clarify how pharmacological and patient-based risk factors interact to alter skeletal muscle performance during statin therapy. It is anticipated that by elucidating these interactions, statin treatment protocols can be more accurately stratified to match desired cholesterol-lowering/cardiovascular outcomes with an individual's potential risk of SAMS.

7.5. Major outcome 4: *The development of statin-induced myalgia is not associated with adverse changes in cardiac or vascular smooth muscle, but the effects of statins on these parameters could vary with lipophilicity*

7.5.1. Summary

The results of this study verify that statin-induced myalgia is not associated with adverse changes in cardiac or vascular smooth muscle performance. Importantly, these findings were not only evident in the work presented in Chapter 5 but were reproduced in Chapter 6. In turn, these results address a major gap in the literature as changes in cardiovascular performance have rarely been investigated in studies of SAMS. While the SIM80 animals showed no signs of impaired cardiovascular integrity, left ventricular compliance and vascular performance in these animals was slightly different to that of the PRAV160 group. Accordingly, these findings add to the growing body of evidence that the effects of statins on cardiovascular parameters may vary between different formulations.

7.5.2. Significance

As mentioned above, this outcome is important as it provides quantitative evidence to support that mild forms of SAMS are not associated with adverse changes in cardiovascular parameters. During rhabdomyolysis, the release of large amounts of intramuscular constituents can induce unfavourable alterations in cardiac performance (i.e. cardiac dysrhythmias) [29, 30], as well as negative changes in haemodynamic parameters [31, 32]. Mild variants of SAMS are not associated with significant increases in serum levels of intramuscular components [33]. Hence, there is a smaller risk of cardiovascular-related

damage in these cases. Nonetheless, the myocardium and blood vessels are still muscle-based structures, and as such they can be directly affected by statin-induced myotoxicity [34, 35]. The findings of this study, however, agree with other reports that these tissues are more resistant to statin-induced myotoxicity compared to skeletal muscle [36]. Therefore, these results support the continued use of statins in cases of statin-induced myalgia (providing affected individuals can tolerate the muscle-related discomfort) [4].

This project has also generated evidence that pravastatin exerts different effects on cardiac and vascular smooth muscle compared to simvastatin, even when administered at an equipotent dose. Studies comparing the effects of hydrophilic and lipophilic statins on cardiovascular parameters have reported conflicting observations [37-39]. Not all investigations have used equipotent doses of statins, and this fact may be contributing to the variability in results. Clarifying the effects of different statins on myocardial and vascular integrity may have significant implications for matching statin treatment to desired cardiovascular outcomes. For instance, if a particular statin is associated with better clinical outcomes in patients following an acute myocardial infarction, then its use in this population may be preferred [37]. In the present investigation, pravastatin appeared to exert slightly better effects on cardiac and vascular performance compared to simvastatin. While these observations need to be verified in models of cardiovascular morbidity, the potential for pravastatin to produce greater cardioprotective benefits could influence future choices for statin prescriptions (particularly if pravastatin is also capable of exerting favourable effects in skeletal muscle).

7.5.3. Future directions

While the findings of this investigation are significant, the results cannot be directly applied to other populations (as young, healthy rats with no cardiovascular disease were used). Other conditions, such as obesity and metabolic syndrome, can induce their own adverse effects on skeletal muscle integrity [16, 40]. Hence, studies using subjects with cardiovascular disease are required to evaluate whether the presence of mild SAMS affects the ability of these medications to improve cardiovascular performance.

It is also recommended that more mechanistic studies are conducted to elucidate the processes responsible for the variable effects of statins on cardiovascular parameters. Again, these investigations should be conducted using models of cardiovascular morbidity (such as heart failure and hypertension) as this will allow for any variation in the effects of different statins to be more easily evaluated. These studies should also be performed using equipotent doses of statins in order to reduce the likelihood of ambiguous results being generated. It is anticipated that the data produced from these studies will clarify whether the prescription of statins should be stratified according to specific cardiovascular outcomes.

7.6. Major outcome 5: *GGPP supplementation (in the form of GGOH) can abrogate statin-induced muscle fatigue* in vivo

7.6.1. Summary

More effective interventions for managing SAMS are required in order to improve patient quality of life, as well as statin compliance. These therapies should protect skeletal muscle without impacting upon the cholesterol-lowering and/or pleiotropic effects of statins. The findings of this study demonstrate that GGPP administration (in the form of GGOH) can

alleviate statin-induced myalgia without significantly impairing cardiovascular performance. The protective effects of GGOH occurred without alterations in *Atrogin-1*, *Pdk4* or *Sod2* expression. Accordingly, this result verified that the expression of these genes is not correlated with statin-induced muscle fatigue.

7.6.2. Significance

This major outcome is significant as it supports that altered GGPP homeostasis is a key factor in the development of SAMS. Several *in vitro* studies have implicated GGPP depletion in statin-induced myotoxicity [41-45]; however, its role *in vivo* was yet to be confirmed. The findings of this project reiterate that elucidating the exact consequences of GGPP depletion/reduced GTPase activity in skeletal muscle will clarify the cellular processes underlying SAMS. Although the findings of this investigation need to be verified by other studies, the ability of GGOH to abrogate statin-induced myotoxicity has significant implications for improving patient compliance with these medications, and thus cardiovascular outcomes.

As noted above, the administration of GGOH in this work was not associated with adverse changes in cardiovascular parameters. Rather, there was evidence that GGOH slightly improved cardiac and vascular function in both control and statin-treated animals. Again, these findings need to be verified by other investigations; nonetheless, they do suggest that the interaction between statins and GGPP in cardiac and vascular smooth muscle may be more complex than previously supposed. Indeed, the notion that the pleiotropic effects of statins are mostly attributable to GGPP depletion has stemmed from investigations focusing on alterations in RhoA activity [46, 47]. However, there are other geranylgeranylated

GTPases (e.g. Rac1) which are affected by statin administration that exert different effects to RhoA in cardiac and vascular smooth muscle [48]. Hence, the impact of statin therapy on GTPase activity, and the biological consequences of any associated changes, require further investigation. Not only will this information clarify the relationship between statins and GTPase activity, but it will also allow for the mechanisms underlying the pleiotropic and adverse effects of these medications to be better understood.

7.6.3. Future directions

As very few studies have assessed the effects of GGPP repletion on skeletal, cardiac and vascular smooth muscle integrity following statin treatment, further investigations are required to validate the findings of this project. Specifically, it is pertinent to assess whether the physiological effects of GGOH administration observed in the present investigation are not altered by the presence of cardiovascular morbidity. This information needs to be established as there is evidence that that GTPases can exert differential effects under pathological and non-pathological conditions [47].

On this note, the exact mechanisms underlying the functional changes induced by GGPP repletion need to be expounded. Specifically, it is important to establish if GGOH administration improves skeletal muscle performance by *directly* reversing statin-induced effects, or whether other (independent) mechanisms are involved. This point is pertinent considering that treatment with GGOH increased force production in the absence of statin-induced muscle fatigue (i.e. the CON + GGOH animals exhibited greater muscle performance compared to the CON group). Additional studies are thus required in order to (i) elucidate the specific cellular changes which underlie statin-induced muscle fatigue and; (ii) determine

if these are directly reversed by the delivery of GGOH. Key parameters to be investigated include changes in GTPase activities and excitation-contraction coupling (see section 7.8.3. for further discussion).

7.7. Major outcome 6: *Validating rodent models of SAMS can significantly reduce inter-study variability, as well as ambiguity surrounding the pathogenesis of these conditions*

7.7.1. Summary

This work has verified that the SIM80 model is suitable for use in pharmacological studies investigating statin-induced myalgia. The results of this project demonstrate that this model is reproducible and produces symptoms which are characteristic of mild SAMS observed in humans. Furthermore, its use has generated important data about the functional, molecular and biochemical changes associated with statin-induced myalgia. Similarly, this model has clarified the influence of lipophilicity and dose on the development of SAMS, as well as the potential of GGPP repletion to prevent statin-induced myotoxicity *in vivo*.

7.7.2. Significance

This outcome is significant as it highlights the importance of validating rodent models of SAMS. By using a reproducible model of statin-induced myalgia, the studies conducted during this project were able to build-upon one another. Thus, this project was able to clarify some of the molecular/biochemical changes associated with statin-induced myalgia (or absence thereof), as well as the influence of pharmacological factors on these processes.

7.7.3. Future directions

At the onset of this project, it was hypothesised that the SIM80 model would induce alterations in muscle physiology which were commonly observed in rodent studies of statin-induced myotoxicity (e.g. muscle atrophy, mitochondrial dysfunction, significant elevations in CK, etc.). Consequently, the gene expression assays and ELISA kits purchased for this project were aimed at assessing these parameters. However, as previously discussed, these factors were found not to be significantly altered by statin treatment. Consequently, further mechanistic studies are required to elucidate the cellular alterations responsible for the functional responses observed in this project (see section 7.8.3. for further discussion).

7.8. Project limitations

This section discusses the key limitations associated with this project. Suggestions outlining how these shortcomings can be addressed in future studies are also provided (as applicable).

7.8.1. Absence of histological and protein expression analyses

Due to time and resource constraints, histological and protein expression analyses were not completed as part of this project. The lack of these assessments, however, is a significant limitation of this work. Indeed, without histological analyses, the absence/presence of overt structural damage in skeletal muscle following statement treatment could not be conclusively determined. While biochemical markers of skeletal muscle damage were measured (i.e. hydroxyproline content, serum myoglobin, CK-MM, etc.), a direct visual evaluation of isolated muscles would have increased the accuracy with which the physical integrity of these

tissues could be described. This point is particularly pertinent for Chapter 3 in which the muscles isolated from the SIM80 rats were referred to as being "damaged" (and the SIM50 muscles as "undamaged"). As histology had not been performed, a definitive presence/absence of tissue injury could not be ascertained. This limitation was addressed in Chapters 4-6 by only using the terms "muscle fatigue" or "reduced functional performance" to describe the physiological changes induced following statin treatment.

Histological assessments would have also clarified the physiological significance of the slight increase in left ventricular mass observed in the SIM80 rats. As this biometric change was not accompanied by any significant alterations in functional performance, or *Mhc-β/Bnp* expression, it was not considered to be clinically significant. Nonetheless, a visual assessment of the left ventricles to validate this assumption would have been beneficial.

The inclusion of protein expression analyses would have enabled the association (or lack thereof) between mitochondrial dysfunction/altered metabolism/oxidative stress and statin-induced muscle myalgia to be more rigorously evaluated. As only the expressions of genes related to these processes were assessed, the potential involvement of these factors in the onset of statin-induced muscle fatigue could not be fully determined. It was only possible to comment on the "association" between these genetic markers and skeletal muscle function. Thus, future studies using the SIM80 model should evaluate changes in the level of proteins related to mitochondrial dysfunction/altered metabolism/oxidative stress in order to build-upon the findings of this work and further elucidate the mechanisms underlying this variant of SAMS.

7.8.2. Potential influence of uncontrolled fasting/fed state on gene expression data

The mRNA expression results presented in Chapter 4 differed from those reported in Chapter 3 (see Appendix C for a tabular summary and comparison of this data). Specifically, the significant differences in mRNA levels between the CON and SIM80 groups reported in Chapter 3 (namely for isolated gastrocnemius muscles) were absent in Chapter 4. As this discrepancy challenged the reproducibility of the SIM80 model, mRNA expression assays were also performed in Chapters 5 and 6 in order to determine if further inconsistencies would be observed. As shown in Appendix C, there was no variation in the SIM80 mRNA levels for Chapters 4-6.

It is possible that not controlling for the fasting/fed states of rodents used in this project may have contributed to the inter-study variability in mRNA levels. Many of the genetic markers investigated in this work are influenced by fasting/feeding (particularly genes related to atrophy/protein degradation and metabolism) [49, 50]. For ethical reasons, animals were not fasted prior to tissue collection/terminal experiments (see Appendix B, section B.2.1., for full details). However, not implementing this control is likely to have introduced within-group heterogeneity into the gene expression data. In turn, this variability may also have reduced the statistical power of some of the comparisons reported in this investigation (i.e. decreased between-group differences and effect sizes). Future investigations evaluating the molecular pathogenesis of SAMS should thus consider using fasted subjects in order to eliminate this potential source of heterogeneity.

7.8.3. Absence of skeletal muscle excitation-contraction coupling studies

It was not within the scope of this project to investigate the effects of statin- or GGOH-treatment on skeletal muscle excitation-contraction coupling (ECC). The absence of ECC studies, however, is a significant shortcoming of this work and limited the comprehensiveness with which the mechanisms underlying the observed changes in force production could be explained. For instance, the muscles isolated from the PRAV160 and SIM80 rats differed not only in terms of overall contractile performance, but also their responsiveness at certain frequencies of the FFC. Using the molecular endpoints evaluated in this project, it was not possible to fully elucidate the reasons for these effects. Thus, assessing ECC-related parameters (e.g. t-tubule system depolarisation, sarcoplasmic reticulum calcium release, intramuscular ATP levels, etc.) will be integral for expounding the findings of this work.

7.8.4. Undetermined reductions in food/water intake and body mass

A final limitation of this work was that the reasons for the reduction in food/water intake and body mass observed in the statin-treated groups were not fully established. In Chapter 3, it was proposed that these biometric changes may have been due to physiological stress caused by the presence of myalgia. However, food/water consumption and body weight gain were also reduced in the SIM40, PRAV160 and SIM+GGOH groups (i.e. those statin-treated groups in which myalgia was absent). This point was briefly considered in Chapters 4 and 6 and it was concluded that these effects were not pathological. Nevertheless, the precise reasons for these biometric changes could not be determined during this project. Accordingly, further work is needed in order to establish the exact causes (and physiological significance) of these effects.

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CHAPTER 8

Conclusion

Preamble

This chapter summarises the key findings and discussions presented in this thesis.

8. Conclusion

Developing more effective strategies for managing SAMS is critical for improving quality of life for affected individuals, as well as cardiovascular outcomes. This research project aimed to address this issue by: (i) clarifying the influence of statin dose and lipophilicity on the pathogenesis of SAMS; (ii) assessing the ability of GGPP administration (in the form of geranylgeraniol) to prevent SAMS *in vivo* and; (iii) evaluating cardiac and vascular smooth muscle performance in the presence of SAMS. By addressing these points, this project has generated novel findings which not only clarify areas of conflict in the literature, but also identify future research directions which will increase current understanding of the pathogenesis and management of SAMS.

Firstly, this project has identified that alterations in genes related to atrophy, oxidative stress, mitochondrial biogenesis, myosin heavy chain isoforms and metabolism are not closely associated with the development of statin-induced myalgia. Alternatively, factors directly affected by GGPP homeostasis are likely to be central to the pathogenesis of this condition. Indeed, the administration of geranylgeraniol was shown to prevent statin-induced muscle weakness/fatigue *in vivo* and may thus be a viable intervention for managing SAMS.

The findings of this project have also clarified that, in the absence of other risk factors for SAMS, statin lipophilicity and dose can significantly affect the myotoxic potential of statins. Specifically, the results support that the risk of SAMS is greater with high-dose therapy versus a low-dose protocol, and more likely when using a lipophilic statin compared to a hydrophilic formulation. Furthermore, this investigation has identified that low-dose or hydrophilic statins can induce beneficial changes in skeletal muscle performance.

Additionally, the results obtained from this project verify previous assumptions that mild SAMS is not associated with adverse alterations in cardiac or vascular smooth muscle performance. Nonetheless, it appears that the effects of statins on cardiovascular parameters may vary with lipophilicity.

Along with these findings, this investigation has also identified several variables which should be considered in future mechanistic studies of SAMS. Firstly, this project has demonstrated the importance of validating rodent models of SAMS, particularly in terms of their reproducibility and representativeness of statin-induced myotoxicity. Dose-response study designs, as well as functional assessments of skeletal muscle performance, are also pertinent for evaluating the physiological significance of any molecular/biochemical alterations which may occur in these models.

Finally, the results generated from this project provide quantitative evidence that each manifestation of SAMS follows a different pattern of development. Hence, future investigations must appreciate that the findings generated when studying a particular form of SAMS cannot be directly applied to other variants. In turn, this realisation will allow for more targeted and effective interventions for SAMS to be identified.

APPENDIX A

The effect of lipophilicity and dose on the frequency of statin-associated muscle symptoms: A systematic review and meta-analysis – Supplementary Data

Preamble

The meta-analysis provided in Chapter 2 referred to Supplementary data which was not presented in the manuscript. For ease of review, the online versions of these files (which can be accessed via the following link: https://doi.org/10.1016/j.phrs.2017.09.013) have been included in this Appendix. An index of the Tables and Figures included in this Appendix is provided on the following page.

A. Supplementary Data

List of Supplementary Tables

List of Supplementary Figures

Figure S1. Funnel plot of the risk ratio of developing muscle symptoms, by the standard error, for randomised controlled trials (clear circles) included in the meta-analysis. Risk ratios are displayed on a logarithmic scale.

Table S1 Search terms used in PubMed and Scopus databases

Search	Search combination	Number of studies generated in Pubmed	Number of studies generated in Scopus
1	lovastatin OR fluvastatin OR pitavastatin OR simvastatin OR atorvastatin OR rosuvastatin OR pravastatin OR cerivastatin AND statin-induced myopathy	151	257
2	lovastatin OR fluvastatin OR pitavastatin OR simvastatin OR atorvastatin OR rosuvastatin OR pravastatin OR cerivastatin AND myalgia	236	2587
3	lovastatin OR fluvastatin OR pitavastatin OR simvastatin OR atorvastatin OR rosuvastatin OR pravastatin OR cerivastatin AND myopathy	1238	2977

Table S2 Details of statin lipophilicity, dose equivalents and systemic bioavailability

Statin type	Lipophilicity Log D class (pH 7.4)‡	Lipophilicity Log P (*N*-octanol/water partition co-efficient) †	Systemic bioavailability (%)†	Atorvastatin equivalent dose^
Lipophilic:				
Atorvastatin	1.00 to 1.25	1.11	12	10 mg
Cerivastatin*	1.50 to 1.75	1.69	60	0.4 mg
Fluvastatin	1.00 to 1.25	1.27	10-35	80 mg
Lovastatin	1.50 to 1.75	1.70	< 5	40 mg
Pitavastatin	1.00 to 1.25	1.49	60	2 mg
Simvastatin	1.50 to 1.75	1.60	< 5	20 mg
Hydrophilic:				
Pravastatin	-0.75 to -1.00	-0.84	18	40 mg
Rosuvastatin	-0.25 to -0.50	-0.33	20	2.5 mg

*Withdrawn in 2001; ‡Information sourced from: White, CM. A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin. J Clin Pharmacol 2002;42:963-970; †Information sourced from: Gazzerro P, Proto MC, Gangemi G, et al. Pharmacological actions of statins: a critical appraisal in the management of cancer. Pharmacol Rev 2012:64:102-146; ^Information sourced from: Veteran Affairs Pharmacy Benefits Management Services Medical Advisory Panel. National Drug Monograph, Pitavastatin (Livalo®). Washington, D.C.: US Departments of Veterans Affairs, 2002. (Accessed September 10 2016, at https://www.pbm.va.gov/clinicalguidance/drugmonographs.asp)

Table S3 Details of subgroup analyses performed in a meta-analysis of statin lipophilicity, dose and muscle symptoms

Parameter	Subgroups
LDL-C entry criteria	LDL-C entry criteria $\geq$ 3.4 mmol L^{-1}
	LDL-C entry criteria < 3.4 mmol L^{-1}
	LDL-C entry criteria both $\geq$ 3.4 and < 3.4 mmol L^{-1}
	LDL-C criteria not specified
Myopathy/CK/statin sensitivity exclusion criteria	Explicit exclusion of individuals with history of myopathy/elevated CK/sensitivity to statins
	No explicit exclusion of individuals with history of myopathy/elevated CK/sensitivity to statins
Lipophilicity	Lipophilic
	Hydrophilic
Dose range*	Prescribed dose < 40 mg
	Prescribed dose $\geq$ 40 mg
Lipophilicity and dose treatment combination	Lipophilic+Low
	Lipophilic+High
	Hydrophilic+Low
	Hydrophilic+High
Median follow-up period	Median follow-up period < 6 m
	Median follow-up period $\geq$ 6 m
Mean participant age	Mean age of participants < 65 y.o
	Mean age of participants $\geq$ 65 y.o
Gender	Only female participants recruited
	Only male participants recruited

*All doses normalised to atorvastatin dose equivalents; CK, creatine kinase; LDL-C, low density lipoprotein; m, months; y.o, years old

Table S4 Quality of study design of randomised controlled trials included in a meta-analysis of statin lipophilicity, dose and muscle symptoms

Study	How were patients randomised?	Blinding of participants and staff	Number of withdrawals reported? (Y/N)	Number of drop-outs reported? (Y/N)	Reasons for withdrawal and/or drop-out stated? (Y/N)	Adverse event monitoring*	Jadad score
4-D[1]	Randomly assigned by computer-generated code	DB	Y	Y	Y	CK measured but specific protocol not specified	5
4S[2]	Randomisation stratified for clinical site but no specific protocol specified	DB	Y	Y	Y	CK measured every 6 w during the first 18 m and every 6 m thereafter	4
A to Z[3]	Randomly assigned to allocation numbers using a blocked randomisation scheme	DB	Y	Y	Y	CK measured prior to treatment, at 1, 4 and 8 m and every 4 m thereafter. If CK > 5 x ULN, repeat measure within 3 d was required. If single CK measurement $\geq$ 10 x ULN with muscle symptoms, or consecutively > 10 x ULN and without muscle symptoms, individual was withdrawn from study	5
ACAPS[4]	Randomised using blocked randomisation with stratification by clinical centre	DB	N	Y	N	Adverse-event data collected but specific protocol not specified	4
AFCAPS/TexCAPS[5]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Extensive safety evaluations were performed prior to treatment, 1 y and at	4

						subsequent-end year visits, but no specific protocol/s specified	
ALLIANCE[6]	Randomised to treatment but no specific protocol specified	NB	Y	Y	Y	Adverse-event data collected every 6 m but specific protocol not specified	2
APATH[7]	Randomised using block randomisation technique	DB	Y	Y	Y	CK measured at baseline and regular intervals but specific protocol not specified	5
APOLLO[8]	Randomisation stratified to LDL-C level, presence or absence of PCI and presence or absence of diabetes but no specific protocol specified	BE	Y	Y	Y	Adverse-event data collected but specific protocol not specified	2
ASA-STAT[9]	Randomised to treatment using a web-based computerised system	DB	Y	Y	Y	CK measured at 6 w and 3 and 6 m	5
ASCOT-LLA[10]	Randomly assigned by computer	DB	N	Y	N	Specific protocol not specified	4
ASEPSIS[11]	Randomised using a computer-generated randomisation sequence	DB	Y	Y	Y	CK measured at 0, 4, 7 and 28 d	5
ASPEN[12]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at baseline and 1, 2, 3, 6, 18, 30 and 42 m	4
ASTRONOMER[13]	Randomisation was centralised and generated by a computer program	DB	Y	Y	Y	CK measured, and adverse side effects information collected, at baseline and every 3 m thereafter	5
AURORA[14]	Randomly assigned in blocks of four	DB	Y	Y	Y	CK measured but specific protocol not specified	4

AVERT[15]	Patients stratified according to whether they had single- or double-vessel disease and randomly assigned to treatments (no specific protocol specified)	NR	Y	Y	Y	CK measured but specific protocol not specified	2
Bak et al.[16]	Randomisation performed by assigning a computer-generated random number to each subject	DB	Y	Y	Y	CK measured every month during treatment period	5
Bays et al.[17]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK, patient-reported adverse signs and symptoms and investigator observations collected but specific protocol not specified	4
Bays et al.2[18]	Study participants were centrally randomised at the study level in 1:1 fashion	DB	Y	N	Y	CK measured and physical examinations performed by specific protocol not specified	5
Beigel et al.[19]	Randomised to treatment using a computer-generated pseudo-random code and random permuted block method	DB	Y	Y	Y	CK measured, and physical examinations performed, but specific protocol not specified	5
Betteridge et al.[20]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, adverse effects assessed, at 0, 6, 13 and 26 w	4
Bone et al.[21]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and adverse events data assessed, at 0, 6 and 12 w	4
Bruckert et al.[22]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and adverse events assessed, at 2 and 6 m	4
CARDS[23]	Randomly assigned by computer-generated code	DB	Y	Y	Y	Adverse clinical events assessed at every follow-up visit. Study treatment	5

						discontinued if repeat CK measured > 10 x ULN	
CARE[24]	Randomly assigned by means of a telephone call to the data centre	DB	Y	N	Y	CK measured but specific protocol not specified	4
Carlsson et al.[25]	Stratified randomisation using a forced block design	DB	Y	Y	Y	CK measured 3 times across 4 m treatment	5
Cash et al.[26]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Adverse-event data collected but specific protocol not specified	4
Chan et al.1[27]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
Chan et al.2[28]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at 0, 4, 8, 12 and 20 w	4
Chan et al.3[29]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured monthly for 2 m then bimonthly thereafter	4
Chan et al.4[30]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
Chua et al.[31]	Randomly assigned using a computer-generated randomisation table	DB	Y	Y	Y	Adverse event monitoring by active query and spontaneous reporting, but specific protocol not specified	5
Cojocaru et al.[32]	Randomised to treatment but no specific protocol specified	NR	Y	Y	Y	CK measured at 0, 3 and 6 m	2
COMETS[33]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured 0 and 6 w	4

CORONA[34]	Randomly assigned using centralised interactive Web-based response system	DB	N	Y	Y	CK measured, and questionnaire on muscle symptoms performed at 6 and 15 m then yearly thereafter	5
Cowan et al.[35]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Adverse-event data collected but specific protocol not specified	4
CRISP[36]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and symptom checklist completed, at 3 and 6 m	4
CSG[37]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and adverse events assessed, every 2 w	4
Cubeddu et al.[38]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
Dadkhah et al.[39]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and patient-reported adverse events recorded, but specific protocol not specified	4
Davidson et al.[40]	Randomisation stratified but no specific protocol specified	DB	Y	Y	Y	Physical examinations performed at screening and 28 d	4
DECREASE-IV[41]	Randomised using a computer algorithm stratified according to hospital	NB	N	Y	N	CK measured but specific protocol not specified	2
Dhamija et al.[42]	Randomisation to treatment using a computer-generated code	DB	Y	Y	Y	Adverse-event data collected but specific protocol not specified	5
DIATOR[43]	Randomised using a computer-generated randomisation list with stratification for participating centres	DB	Y	Y	Y	Specific protocol not specified	5
Durazzo et al.[44]	Randomised to treatment using a computer algorithm	DB	Y	Y	Y	CK measured during the hospital stay	5

ESG[45]	Balanced randomisation accomplished using computer-generated randomisation schedule	DB	Y	Y	Y	CK measured, and physical examinations performed, at baseline and 12 w	5
ESG2[46]	Randomised to treatment but no specific protocol specified	DB	Y	N	Y	CK measured but specific protocol not specified	4
ESG3[47]	Balanced randomisation accomplished using computer-generated randomisation schedule	DB	Y	Y	Y	CK measured, and physical examinations performed, but specific protocol not specified	5
ESP[48]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
EXCEL[49]	Randomisation stratified but no specific protocol specified	DB	N	N	N	CK measured but specific protocol not specified	3
EZET-ATOR[50]	Randomised to treatment but no specific protocol specified	DB	Y	N	Y	CK measured, and safety evaluated through patient reports and investigator observations, but specific protocol not specified	4
FACS[51]	Randomised to treatment but no specific protocol specified	DB	Y	Y	N	CK measured at 30 d	4
FLARE[52]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK levels of all patients to be within reference range at randomisation. CK measured at each attendance	4
Fogari et al.[53]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Not required	CK measured, and adverse events assessed, at 6, 12 and 26 w	4

						CK measured at baseline and follow-up.	
GAUSS-3[54]	Randomisation performed using an interactive web-based or voice recognition system	DB	Y	Y	Y	Pre-specified safety and tolerability outcomes included with incidence of muscle-related adverse effects	5
Gentile et al.[55]	Randomised to treatment but no specific protocol specified	NB	Y	Y	Y	CK measured, and physical examination performed, before and after treatment	2
Ghirlanda et al.[56]	Randomised to treatment but no specific protocol specified	DB	N	N	N	CK measured at 0 w, weekly for 1st month and monthly thereafter. Physical examination performed at 6 and 12 w	2
GISSI-P[57]	Central randomisation made in separate blocks for each centre	NR	N	Y	Y	Specific protocol not reported	3
GISSI-HF[58]	Randomised using a computerised telephone randomisation system	DB	Y	Y	Y	CK measured, and adverse effects assessed, at 1, 3, 6 and 12 m	5
GREACE[59]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at 6, 12 and 24 w and every 6 m thereafter. Myalgia (muscle symptoms) evaluated but specific protocol not specified	3
Hommel et al.[60]	Randomised to treatment but no specific protocol specified	DB	Y	N	Y	CK measured but specific protocol not specified	4
HOPE-3[61]	Randomised to treatment but no specific protocol specified	DB	Y	N	N	Specific protocol not reported	3
Hunninghake et al.[62]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Physical examination at baseline and 1, 2, 4, 8 and 12 w	4

Hunninghake et al.2[63]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and physical examinations performed, but specific protocol not specified	4
HYRIM[64]	Patients randomly assigned according to a 2 × 2 factorial design	DB	N	N	N	CK measured but specific protocol not specified	4
Jacobson et al.[65]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and data on possible adverse events collected, at 6 and 12 w	4
John et al.[66]	Randomisation stratified by age using a computer-generated code	DB	Y	Y	Y	CK measured but specific protocol not specified	5
JUPITER[67]	The central telephone randomisation system used a minimisation algorithm to balance the treatment groups with respect to eligibility criteria and other major prognostic factors	DB	Y	Y	Y	CK measured in patients reporting unexplained muscle symptoms or concomitant use of non-study statin	5
KAPS[68]	Randomisation performed using an interactive voice-response system and was stratified according to centre	DB	N	N	N	Potential adverse events assessed at 13 w, 6 m and every six months thereafter	4
Kennedy et al.[69]	Randomised to treatment using a random number generator	DB	Y	Y	Y	CK measured at 5 and 8 w	5
Konduracka et al.[70]	Randomised to treatment but no specific protocol specified	NR	N	Y	Y	CK measured at 0 and 6 w and every 3 m thereafter	2
Krysiak & Okopień[71]	Randomised to treatment but no specific protocol specified	NR	Y	Y	Y	CK measured but specific protocol not specified	2
Krysiak et al.[72]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at 0, 4 and 12 w	4

Krysiak et al.2[73]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Adverse-event data collected twice monthly	4
Krysiak et al.3[74]	Randomised to treatment using a computer program	DB	Y	Y	Y	CK measured but specific protocol not specified	5
Krysiak et al.4[75]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Adverse-event data collected but specific protocol not specified	4
LCAS[76]	Randomisation stratified but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
Lewis et al.[77]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
Lijnen et al.[78]	Randomised to treatment but no specific protocol specified	NR	Y	Y	Y	CK measured but specific protocol not specified	2
LIPID[79]	Randomised to treatment but no specific protocol specified	DB	N	N	N	CK measured every 6 w in 1st y and every 3 m thereafter	3
LIPS[80]	Randomisation and stratification to qualifying event and clinical centre but no specific protocol specified	DB	N	Y	N	Data on hospital admissions and serious events obtained ever 6 m	3
LISA[81]	Randomly assigned at each centre according to medication pack numbers using block randomisation	DB	Y	Y	Y	CK measured but specific protocol not specified	5
LRT[82]	Randomised to treatment but no specific protocol specified	DB	N	Y	Y	CK measured at 20, 36 and 52 w	4
Lye et al.[83]	Randomised to treatment but no specific protocol specified	DB	Y	N	Y	Adverse-event data collected but specific protocol not specified	4

Lynch et al.[84]	Randomised to treatment but no specific protocol specified	DB	Not Required	Not Required	Not Required	CK measured daily for 14 d	4
MAAS[85]	Randomised to treatment but no specific protocol specified	DB	Y	N	N	CK measured at baseline and 4, 12, 18 and 26 w after angioplasty	3
MARS[86]	Randomisation stratified but no specific protocol specified	DB	Y	N	Y	Specific protocol not specified	4
MEGA[87]	Randomisation stratified to gender, smoking status and TC level but no specific protocol specified	DB	Y	N	Y	Specific protocol not specified	4
METEOR[88]	Patients were randomly by computerised randomisation following the permuted method	DB	Y	Y	Y	CK measured, adverse events assessed, at 1, 3 and 6 m and every 6 m thereafter	5
Meyers et al.[89]	Randomised to treatment using a computer generated block design	DB	Y	Y	Y	CK measured at 0, 6 and 12 w	5
MIRACL[90]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured every 3 m. Adverse events assessed by means of standard questions	4
Mohebbi et al.[91]	Patients randomly assigned using a permuted-block randomization	DB	N	N	N	Patients asked about myalgia, muscle spasms and musculoskeletal pain	3
Morgan et al.[92]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and adverse events assessment, at visit 0, 4, 6, 8 and 10	4
MRC/BHF HPS[93]	Randomly assigned with stratification by centre but no specific protocol specified	DB	Y	Y	Y	Specific protocol not specified	4
Napoli et al.[94]	Randomised to treatment but no specific protocol specified	NR	Y	N	Y	CK measured, and adverse event data collected, at 3, 6, 12, 18 and 24 m	2

NPSG[95]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Patients questions about adverse events and CK measurements	4
OCS[96]	Randomisation was by telephone to the Clinical Trial Service Unit, Oxford, with treatments allocated using a computer minimisation algorithm	DB	Y	Y	Y	Patients questioned for muscle problems at 8 w then at 12 w intervals for 1 y and then at 24 w intervals thereafter. CK measured if muscle pain was reported	5
PACT[97]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Adverse events assessed at 4 w	4
Päivä et al.[98]	Randomised using a block-of-18 randomisation scheme	DB	Y	Y	Y	CK measured at 0, 1, 2, 4 and 8 w	5
Panahi et al.[99]	Randomised to treatment but no specific protocol specified	DB	N	N	N	CK measured but specific protocol not specified	2
Panichi et al.[100]	Randomised to treatment but no specific protocol specified	DB	N	Y	Y	CK measured but specific protocol not specified	4
Parker et al.[101,102]	Randomised to treatment but no specific protocol specified	DB	Not required	Not required	No required	Patients contacted weekly to inquire about muscle symptoms using the Brief Pain Inventory, Short Form (pain - severity and -interference scores calculated) and CK measured	4
PEARL[103]	Randomised using a minimisation method with biased-coin assignment	NB	Y	Y	Y	CK measured but specific protocol not specified	3
PLAC I[104]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4

PMSGCRP[105]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4
PMSGD[106]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at baseline and 16 w	4
Pollo-Flores et al.[107]	Randomised using a computer-generated simple random sample list	TB	Y	Y	Y	Adverse-event data collected but specific protocol not specified	5
PROCAS[108]	Randomised to treatment using a computer program	DB	Y	Y	Y	CK measured once every 12 m	5
PROSPER[109]	The randomisation sequence was generated with a computerised pseudorandom number generator and consisted of balanced blocks of size four	DB	Y	Y	Y	CK measured formally at 3 m	5
PTT[110]	Randomised to treatment but no specific protocol specified	DB	N	N	N	Patients questioned about adverse experiences at 3 and 6 m	3
Raskin et al.[111]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and patients questioned about adverse effects, at baseline, 2 w intervals for first 4 w and 4 w intervals during remaining 20 w	4
REGRESS[112]	Randomised to treatment but no specific protocol specified	DB	N	N	N	Patients to report any adverse reactions and all other clinical events	3
RIGHT[113]	Randomised to treatment but no specific protocol specified	DB	N	N	N	CK measured but no specific protocol not specified	3
ROSUV[114]	Randomised to treatment but no specific protocol specified	DB	Y	N	N	CK measured, and safety analysis performed, but specified protocol not	3

ROSUVATOR[115]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and physical examinations performed, but specific protocol not specified	4
Saito et al.[116]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at 4 and 8 w	4
SALTIRE[117]	A blinded study coordinator randomly assigned patients to treatment with the use of a locked computer program	DB	Y	N	Y	CK measured, and adverse events assessed, at 2 and 6 m and every 6 m thereafter	4
Sano et al.[118]	Randomised to treatment using a random permuted block treatment assignment stratified by site	DB	Y	Y	Y	CK measured, and a symptom checklist (with specific queries for muscle pain, tenderness or weakness) completed, but specific protocol not specified	5
Santinga et al.[119]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured 0, 48 and 96 w	4
SHARP[120]	Randomised using a minimised randomisation computer algorithm	DB	N	N	N	CK measured, and information on unexplained muscle pain, collected at 2 m, 6 m and 6 m thereafter	4
Simsek et al.[121]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	Adverse event data collected but specific protocol not specified	4
SPARCL[122]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and adverse events assessed, but specific protocol not specified	4

STATCOPE[123]	Randomised to treatment but no specific protocol specified	NR	Y	Y	Y	Adverse event data collected but specific protocol not specified	2
Stegmayr et al.[124]	Stratified randomisation using a telephone call to the study data centre	NB	Y	Y	Y	CK measured at baseline and at 1, 3, 6, 12, 18, 24, 30 and 36 m	3
Stein et al.[125]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and adverse event assessed, but specific protocol not specified	4
STOMP[126]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at baseline and at 6 m. Muscle complaints assessed at baseline, 3 and 6 m using predefined questionnaire	4
Strey et al.[127]	Randomised to treatment but no specific protocol specified	DB	Y	N	Y	CK measured but specific protocol not specified	4
Taneva et al.[128]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured at baseline, after 24 h, at 1 and 6 w, and 36 hours after final intake of study drug	4
Udawat and Goyal[129]	Randomised to treatment but no specific protocol specified	NB	Y	N	Y	CK measured if patient had symptoms of myopathy	2
UK-HARP-I[130]	Randomisation was by telephone to the Clinical Trial Service Unit	NR	Y	Y	Y	CK measured at 3 and 12 m. Patients asked about muscle pain, weakness or serious adverse experiences at 1, 3, 6 and 9 m and 1 y	3
Urso et al.[131]	Randomised to treatment but no specific protocol specified	DB	N	N	N	CK measured at baseline and 4 w	3

Villegas-Rivera et al.[132]	Randomised to treatment in blocks with a parallel sequence through a randomised computer-based list generated by a different researcher unaware of the drugs given	DB	Y	Y	Y	CK measured but specific protocol not specified	5
Wang et al.[133]	Randomisation stratified but no specific protocol specified	DB	N	N	N	CK measured at 0, 4 and 8 w. Physical examination at screening, baseline and termination	3
Wiklund et al.[134]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured, and physical examinations performed at baseline and 2, 4, 8 and 12 w	4
Wiklund et al.2[135]	Randomised to treatment using a computer-generated randomisation code	DB	Y	Y	Y	CK measured but specific protocol not specified	5
WOSCOPS[136]	Randomised to treatment but no specific protocol specified	DB	Y	Y	Y	CK measured but specific protocol not specified	4

*Evaluation not included in numerical scoring. Abbreviations: BE, blinded end-point; CK, creatine kinase; d, days; DB, double-blind; h, hours; m, months; NB, no-blinding; NR, not reported/specified; TB, triple-blind; ULN, upper limit of normal; w, weeks; y, year

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Table S5 Baseline characteristics of randomised controlled trials included in a meta-analysis of statin lipophilicity, dose and muscle symptoms

Study	Participant details	Study Type	Primary endpoint	Statin (Type)	Dose (mg)	Atorvastatin equivalent dose (mg)	Median follow-up (years)
4-D[1]	T2DM, receiving maintenance haemodialysis (< 2 y); female 46%; mean age 65.7 y.o; LDL-C 2.1-4.9, TG < 11.3 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Death from cardiac causes, nonfatal MI and stroke	Ator (L)	20	20	3
4S[2]	History of AP or acute MI; female 19%; mean age 59.3 y.o; TC 5.5 - 8.0, TG < 2.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Total mortality	Simv (L)	20	10	5.4
A to Z†[3]	Non–ST-elevation ACS or ST-elevation MI; female 30%; mean age 61.0 y.o; TC $\leq$ 6.48 (mmol L^{-1}); excluded if had prior history of nonexercise-related CK elevations or nontraumatic rhabdomyolysis	R, DB, PC (P)	CVD death, nonfatal MI, readmission ACS and stroke	Simv (L)	40 or 80	20 or 40	1.98
ACAPS[4]	Early carotid atherosclerosis, moderately elevated LDL-C; female 49%; mean age 61.7; LDL-C 4.1-5.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in mean maximum intimal-medial thickness in carotid arteries	Lov (L)	20 or 40	5 or 10	2.84
AFCAPS/TexCAPS[5]	No prior history, signs or symptoms of definite MI, angina, atherosclerotic CVD, claudication, CVA or TIA; female 15%; mean age 58.0 y.o; TC 4-7-6.8, LDL-C 3.4-4.9, HDL-C < 1.2, TG < 4.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	First major coronary event	Lov (L)	20 or 40	5 or 10	5.2

ALLIANCE[6]	Known CHD, hyperlipidemia; female 18%; mean age 61.2 y.o; LDL-C 2.8-6.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	Pr, R, UC (P)	First occurrence of a primary cardiovascular event	Ator (L)	10, 20, 40 or 80	10, 20, 40 or 80	4.5
APATH[7]	PAH or CTEPH; female 66%; mean age 36.0 y.o; excluded if history of CK > 5xULN	R, DB, PC (P)	Change in 6-minute walking distance	Ator (L)	10	10	0.46
APOLLO[8]	Undergone elective PCI (coronary plaques); female 26%; mean age 71.4 y.o; LDL-C < 3.6, TG < 4.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	PROBE (P)	Change in the MLD and average lumen diameter of non-target lesions	Rosuv (H)	2.5 or 5	10 or 20	2
ASA-STAT[9]	PAH; female 86%; mean age 50.5 y.o; excluded if CK > 1.5xULN at screening	R, DB, PC (P)	Change in 6-minute walking distance	Simv (L)	40	20	0.5
ASCOT-LLA[10]	HT, $\geq$ 3 CVD risk factors; female 19%; mean age 63.0 y.o; TC $\leq$ 6.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Non-fatal MI and fatal CHD	Ator (L)	10	10	3.3
ASEPSIS[11]	New proven or suspected infection; female 49%; mean age 63.4 y.o; Excluded if history of myopathy or rhabdomyolysis	R, DB, PC (P)	Progression rate of sepsis to severe sepsis	Ator (L)	40	40	1
ASPEN[12]	T2DM; female 34%; mean age 61.1 y.o; LDL-C 3.6-4.1, TG < 6.9 (mmol L^{-1}); excluded if history of CK > 3xULN or taking drugs with increased risk of rhabdomyolysis	R, DB, PC (P)	CVD, nonfatal MI, nonfatal stroke, recanalisation, CAB surgery, resuscitated cardiac arrest and worsening/unstable angina requiring hospitalisation	Ator (L)	10	10	4

ASTRONOMER[13]	Mild to moderate aortic stenosis (asymptomatic); female (NR); age 18-62 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in aortic stenosis progression	Rosuv (H)	40	160	3.5
AURORA[14]	Undergoing maintenance haemodialysis; female 38%; mean age 64.2 y.o; excluded if CK > 3xULN at baseline	R, DB, PC (P)	Death from cardiovascular causes, non-fatal MI or nonfatal stroke	Rosuv (H)	10	40	3.8
AVERT[15]	Stable CAD; female 16%; mean age 58.5 y.o; LDL-C $\geq$ 3.0, TG $\leq$ 5.6 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	O, R, UC (P)	Ischaemic events	Ator (L)	80	80	1.5
Bak et al.[16]	Primary hypercholesterolemia without CVD; female 0%; mean age 55.1 y.o; TC 6.5-8.0, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, HDL-C, TG, LDL-C and apo-lipoproteins A1 and B	Prav (H)	20	5	0.5
Bays et al.[17]	Primary hypercholesterolemia; female 53%; mean age 55.5 y.o; LDL-C 3.8-6.5, TG $\leq$ 4.0 (mmol L^{-1}); excluded if CK > ULN at screening	R, DB, PC (P)	Change in LDL-C	Simv (L)	10, 20, 40 or 80	5, 10, 20 or 40	0.31
Bays et al.2[18]	Overweight, mixed dyslipidaemia; female 53%; mean age 54.6 y.o; LDL-C 3.4-7.3, TG 1.7-6.2, HDL-C $\leq$ 1.6 (mmol L^{-1}); excluded if CK > 3xULN at screening	R, DB, PC (P)	Change in Apo B-100	Ator (L)	10	10	0.15
Beigel et al.[† 19]	Primary hypercholesterolemia, $\geq$ 2 coronary risk factors; female 28%; mean age 55.8 y.o; TC 5.2-7.8, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, TG, HDL-C, ALT, GGT, AST and CK	Prav (H)	20 or 40	5 or 10	0.5
Betteridge et al.[20]	Uncomplicated primary hypercholesterolemia, evidence of CHD, $\geq$ 2 CVD risk factors; female 56%; mean age 54.4 y.o; LDL-C $\geq$ 3.4, TG $\leq$ 4.0 (mmol L^{-1}); excluded if history of muscular abnormalities	R, DB, PC (P)	Change in LDL-C	Ceriv (L) or	0.025, 0.05, 0.1,	0.625, 1.25, 2.5, 5 or 10	0.23

				Simv (L)	0.2 or 20		
Bone et al.[21]	Lumbar (L1–L4) spine bone mineral density T-score between 0 and -2.5, moderately elevated; female 100%; mean age 58.8 y.o; LDL-C 3.4-4.9 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in lumbar (L1–L4) spine bone mineral density	Ator (L)	10, 20, 40 or 80	10, 20, 40 or 80	1
Bruckert et al.[22]	Primary hypercholesterolemia; female 75%; mean age 75.5 y.o; TC $\geq$ 6.5, LDL-C $\geq$ 4.1, TG $\leq$ 4.6 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	Pr, R, DB, PC (P)	Change in TC, LDL-C, TG and HDL-C	Fluv (L)	80	10	0.5
CARDS[23]	T2DM, no documented CVD history, without elevated LDL-C; female 32%; mean age 61.7 y.o; LDL-C $\leq$ 4.14; TG < 6.78 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	First acute CHD event, coronary revascularisation procedures or stroke	Ator (L)	10	10	3.9
CARE[24]	Acute MI 3-20 months before randomisation, LVEF $\geq$ 25% no symptomatic CgHF; female 14%; mean age 59.0 y.o; TC < 4.0, BGL < 12.2 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Death from CHD or a symptomatic nonfatal MI	Prav (H)	40	10	5
Carlsson et al.[25]	Asymptomatic adult children of persons with late-onset Alzheimer's disease, without cognitive impairment themselves; female 75%; mean age 53.5 y.o; excluded if history of adverse reactions to statins or elevated CK	R, DB, PC (P)	Change in cerebrospinal fluid amyloid-β levels and cognition	Simv (L)	40	20	0.33
Cash et al.[26]	Primary biliary cirrhosis, hypercholesterolemia; female 95.2%; mean age 55.0 y.o; TC > 5.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in BMI, blood pressure, glucose, liver function, lipid profile, immunoglobulin levels,	Simv (L)	20	10	1

			endothelial function, anti-oxidant status and vascular compliance				
Chan et al.1[27]	Elderly, HT, hypercholesterolemia, no other CVD risk factors; female 57%; mean age 75.0 y; TC 6.5-10.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	10	2.5	0.5
Chan et al.2[28]	Primary hypercholesterolemia; female 54%; mean age 74.1 y.o; TC > 6.5, TG < 3.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	10	2.5	0.38
Chan et al.3[29]	Elderly, HT, primary hypercholesterolemia; female 49%; mean age 76.5; TC > 6.5, TG < 3.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in lipid, glucose and fasting insulin	Prav (H)	15	3.75	1
Chan et al.4[30]	Elderly, hypercholesterolemia; female 50%; mean age 74.5 y.o; LDL-C 6.5-10.4, TG < 3.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Simv (L)	10	5	0.25
Chua et al.[31]	Mild-to-moderate chronic plaque psoriasis; female 60.7%; mean age 41.0 y.o; excluded if history of any myopathy or elevated CK	R, RB, PC (P)	Change in Psoriasis Area and Severity Index (PASI) score and achievement of PASI-50	Ator (L)	40	40	0.5
Cojocaru et al.[32]	Rheumatoid arthritis; female 86%; mean age 58.8 y.o; excluded if history of adverse reactions to statins, myositis or CK > 2xULN	R, UC (P)	Change in disease activity and frequency/severity of adverse events	Simv (L)	20	10	0.5

COMETS[† 33]	Metabolic syndrome; female 64%, mean age 57.7 y.o; excluded if history of serious reactions to statins or CK > 3xULN	R, DB, PC (P)	Change in LDL-C	Ator (L) or Rosuv (H)	10	10 or 40	0.12
CORONA[34]	Ischaemic or systolic HF; female 24%; mean age 73 y.o; excluded if history of statin-induced myopathy or chronic muscle disease	R, DB, PC (P)	Death from cardiovascular causes, non-fatal MI or nonfatal stroke	Rosuv (H)	10	40	2.7
Cowan et al.[35]	Stable asthma; female 67%; mean age 45.0 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (CO)	'Minimum' inhaled corticosteroid (ICS) dose requirement	Simv (L)	40	20	0.08
CRISP[36]	Elderly; female 71%; mean age 71.2 y.o; LDL-C 4.1-5.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	Pr, R, DB, PC (P)	Change in health-related quality of life including physical functioning, sleep behaviour, social support, depression, cognitive function and health perception	Lov (L)	20 or 40	5 or 10	0.5
CSG[37]	Healthy or definite atherosclerotic disease or $\geq$ 2 CVD risk factors; female 38%; mean age 56.7 y.o; LDL-C $\geq$ 3.4 (mmol L^{-1}); excluded if history of elevated CK	R, DB, PC (P)	Change in LDL-C levels	Ceriv (L)	0.4 or 0.8	10 or 20	0.15
Cubeddu et al.[38]	Dyslipidaemia; female 45%; mean age 46.5 y.o; LDL-C 3.6-4.9 (mmol L^{-1}); excluded if history of CK > 2xULN	R, DB, PC (P)	Change in LDL-C levels	Ator (L)	10	10	0.25

Dadkhah et al.[39]	Sildenafil non-responders, erectile dysfunction, hypercholesterolemia; female 0%; mean age 62.9 y.o; TC < 5.2 mmol L^{-1}, LDL-C < 4.1 (mmol L^{-1}); excluded if CK > 25%xULN at screening	R, DB, PC (P)	Change in erectile function	Ator (L)	40	40	0.23
Davidson et al.[40]	Moderate hypercholesterolemia; female 46%; mean age 56.5 y.o; LDL-C 4.1-5.7, TG $\leq$ 3.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C	Lov (L)	10	2.5	0.08
DECREASE-IV[41]	Scheduled for elective non-cardiovascular surgery, estimated risk perioperative cardiovascular event of 1-6%; female 40%; mean age 64.0 y.o; no explicit myopathy/CK exclusion criteria	Pr, R, DB, PC (P)	Cardiac death and nonfatal MI	Fluv (L)	80	10	0.09
Dhamija et al.[42]	Mildly to moderately severe acute exacerbation of ulcerative colitis; female 41%; mean age 40.3 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in Partial Mayo Score (PMS)	Ator (L)	20	20	0.15
DIATOR[43]	T1DM; female 40%, mean age 29.9 y.o; LDL-C < 3.9 (mmol L^{-1}); excluded if CK > 5xULN at screening	R, DB, PC (P)	Change in fasting serum c-peptide	Ator (L)	80	80	1.5
Durazzo et al.[44]	Undergoing elective non-cardiac arterial vascular surgery; female 21%; mean age 67.2 y.o; no explicit myopathy/CK exclusion criteria	Pr, R, DB, PC (P)	Death from cardiac cause, nonfatal MI, unstable angina and stroke	Ator (L)	20	20	0.12
ESG[45]	Primary hypercholesterolemia; female 43%, mean age 57.6 y.o; LDL-C 3.8-6.5, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C	Simv (L)	10, 20 , 40 or 80	5, 10, 20 or 40	0.23

ESG2[46]	Primary hypercholesterolemia; female 62%; mean age 57.0 y.o; LDL-C 3.8-6.5, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C	Lov (L)	10, 20 or 40	2.5, 5 or 10	0.23
ESG3[47]	Primary hypercholesterolemia; female 52%; mean age 54.3 y.o; LDL-C 3.8-6.5, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C	Prav (H)	10, 20 or 40	2.5, 5 or 10	0.23
ESP[48]	No CAD risk factors and LDL-C > 4.9 or $\geq$ 2 standard CAD risk factors and LDL-C 4.1-4.9 (mmol L^{-1}); female 51%; mean age 68.0 y.o; excluded if history of abnormal CK levels	R, DB, PC (P)	Change in TC, HDL-C, LDL-C and TG	Prav (H)	20	5	0.23
EXCEL[49]	Moderate hypercholesterolemia; female 41%; mean age 68.0 y.o; TC 6.2-7.8, LDL-C $\geq$ 4.1, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C, HDL-C and TG	Lov (L)	20, 40 or 80	5, 10 or 20	0.92
EZET-ATOR[50]	Primary hypercholesterolemia; female 57%; mean age 57.5 y.o; LDL-C 3.8-6.5, TG $\leq$ 9.1 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Reduction in LDL-C	Ator (L)	10, 20, 40 or 80	10, 20, 40 or 80	0.25
FACS[51]	ACS; female 32%; mean age 62.1 y.o; excluded if had history of muscle disease or CK $\geq$ 5xULN	R, DB, PC (P)	Change in C-Reactive protein, interleukin-6 and PAPP-A/proMBP	Fluv (L)	80	10	0.08
FLARE[52]	Symptomatic or ischemia-producing coronary lesions suitable for balloon angioplasty; female 17.5%; mean age 60.5 y.o; LDL-C < 6.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Absolute change in MLD post-PTCA to follow-up	Fluv (L)	80	10	0.78

Fogari et al.[53]	Mild hypercholesterolemia, ≥ 2 CHD risk factors; female 50%; mean age 55.2 y.o; TC 5.2-6.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, HDL-C, LDL-C and TG	Prav (H)	20	5	0.5
GAUSS-3‡ [54]	Hypercholesterolemia, history of intolerance to ≥ 2 statins; female 50%; mean age 60.7 y.o; LDL-C ≥ 2.6 (mmol L^{-1}); >80% patients had history of intolerance to ≥ 3 statins	R, DB, PC (CO)	Change in LDL-C	Ator (L)	20	20	0.19
Gentile et al.[55]	T2DM with hypercholesterolemia; female 32%; mean age 59.0 y.o; LDL-C > 4.2, TG < 4.5 (mmol L^{-1}); excluded if CK > 3xULN at screening	R, O, PC (P)	Change in LDL-C	Ator (L), Lov (L), Prav (H) or Simv (L)	10 or 20	5 or 10	0.46
Ghirlanda et al.* [56]	Hypercholesterolemia, ≥ 2 CHD risk factors; female 40%; mean age 46.7 y.o; TC > 5.7, TG < 3.95 (mmol); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in coenzyme Q10, TC, HDL-C, LDL-C and TG	Prav (H) or Simv (L)	20	5 or 10	0.25
GISSI-P† [57]	Mild hypercholesterolemia, MI; female 14%; mean age 59.9 y.o; TC 5.2-6.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, OT, UC (P)	Cumulative rate of total mortality, non-fatal MI stroke, cumulative rate of cardiovascular mortality, MI and non-fatal stroke	Prav (H)	20	5	1

GISSI-HF[58]	CHF; female 23%; mean age 68.0 y.o; excluded if CK > ULN at baseline	R, DB, PC (P)	Time to death, and time to death/admission to hospital for cardiovascular reasons	Rosuv (H)	10	40	3.9
GREACE[59]	CHD; female 22%; mean age 58.5 y; LDL-C > 2.6, TG < 4.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, UC (P)	Death, nonfatal acute MI, unstable angina, CgHF, revascularisation and stroke	Ator (L)	10, 20, 40 or 80	10, 20, 40 or 80	3
Hommel et al.[60]	T1DM, nephropathy, hypercholesterolemia; female 43%; mean age 38.0 y.o; TC $\geq$ 5.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C, TG and apolipoprotein A-I and B	Simv (L)	10 or 20	5 or 10	0.23
HOPE-3[61]	$\geq$1 CVD risk factors; female 46.3%; mean age 65.8; no explicit myopathy CK/exclusion criteria	R, DB, PC (P)	Death from cardiovascular causes, nonfatal MI or nonfatal stroke, resuscitated cardiac arrest, heart failure, and revascularisation	Rosuv (H)	10	40	5.6
Hunninghake et al.[62]	Primary hypercholesterolemia; female 22%; mean age 51.1 y.o; TG $\leq$ 2.82 mmol L^{-1}; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	5, 10 or 20	1.25, 2.5 or 5	0.23

Hunninghake et al.2[63]	Hypertriglyceridemia; female 40%; mean age 56.1 y.o; TG 3.4-9.0 (mmol L^{-1}); excluded if history of elevated CK	R, DB, PC (P)	Change in TG	Rosuv (H)	5, 10, 20, 40 or 80	20, 40, 80, 160 or 320	0.12
HYRIM** [64]	HT; female 0%; mean age 57.2 y.o; TC 4.5-8.0, TG < 4.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in carotid intima-media thickness	Fluv (L)	40	5	4
Jacobson et al.[65]	Primary hypercholesterolemia; female 39%; mean age 56.5 y.o; LDL-C > 4.1, TG < 4.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG levels	Prav (H)	20	5	0.23
John et al.[66]	Confirmed chronic obstructive pulmonary disease without hypercholesterolemia; female 28%; mean age 64.5 y.o; TC < 6.5 mmol L^{-1}; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in aortic pulse wave velocity	Simv (L)	20	10	0.12
JUPITER[67]	Apparently healthy, high-sensitivity C-reactive protein levels $\geq$ 2.0 mg/L; female 38%; mean age 66.0 y.o; LDL-C < 3.4 (mmol L^{-1}); excluded if CK > 3xULN at screening	R, DB, PC (P)	First major cardiovascular event	Rosuv (H)	20	80	1.9
KAPS[68]	Severe atherosclerotic disease; female 0%; mean age 57.4 y.o; LDL-C $\geq$ 4.0, TC < 7.5 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Rate of carotid atherosclerotic progression	Prav (H)	40	10	3
Kennedy et al.[69]	Hyperlipidaemia, documented myalgias to $\geq$ 1 statin; female 0%; mean age 64.5 y.o; LDL-C < 4.14 (mmol L^{-1}); excluded if history of rosuvastatin-induced myalgia (but not myalgia with other statins) or CK > 1000 U/mL at screening	R, DB, PC (CO)	Change in LDL-C	Rosuv (H)	5	20	0.15

Konduracka et al.[70]	T1DM (> 10 y) without CHD or arterial HT, hypercholesterolemia; female 54.9%; mean age 36.3 y.o; LDL-C $\geq$ 2.6 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, PC (P)	Change in endothelium-dependent flow-mediated vasodilation of brachial artery	Ator (L)	40	40	0.5
Krysiak & Okopień[71]	Lower extremity arterial stenosis, family history of CAD, isolated hypertriglyceridemia; female 43%; mean age 48.5 y.o; TC > 5.2; LDL-C < 1.5; TG 2.3-5.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, PC (P)	Change in plasma lipids, glucose homeostasis markers, plasma C-reactive protein and lymphocyte cytokine release	Simv (L)	80	40	0.25
Krysiak et al.[72]	T2DM with mixed dyslipidaemia; female 43%; mean age 53 y.o; TC > 5.2, LDL-C > 3.4, TG > 1.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Effect on monocyte and lymphocyte cytokine release and low-grade inflammation	Simv (L)	40	20	0.25
Krysiak et al.2[73]	Asymptomatic atherosclerosis, primary hypercholesterolemia; female 45%; mean age 53.2 y.o; TC > 2.6, LDL-C > 3.4, TG < 1.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in plasma lipids and haemostatic cardiovascular risk factors	Simv (L)	40	20	0.25
Krysiak et al.3[74]	Recently diagnosed and untreated isolated hypercholesterolemia; female 45%; mean age 51.0 y.o; TC > 5.2, LDL-C > 3.4, TG < 1.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in lymphocyte cytokine release, high-sensitivity C-reactive protein and intercellular adhesion molecule 1	Simv (L)	40	20	0.23

Krysiak et al.4[75]	Isolated hypercholesterolemia; female 41%; mean age 51.5 y.o; TC > 5.2, LDL-C > 3.4,TG < 1.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in plasma free fatty acids, high-sensitive C-reactive protein and adipokines	Simv (L)	40	20	0.23
LCAS[76]	CHD, mild-to-moderate hypercholesterolemia, angiographic evidence of $\geq$ 1 coronary atherosclerotic lesion, untreated by angioplasty and <100% occluded; female 19%; mean age 58.8 y.o; LDL-C 3.0-4.9; TG $\leq$ 3.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in MLD of qualifying lesions	Fluv (L)	40	5	2.5
Lewis et al.[77]	Hypercholesterolemia, $\geq$ 6-month history of compensated chronic liver disease; female 48%; mean age 49.8 y.o; LDL-C > 2.6; TG < 4.5 (mmol L^{-1}); exclude if CK > 3xULN at screening	Pr, R, DB, PC (P)	Change in LDL-C and ALT	Prav (H)	80	20	0.69
Lijnen et al.[78]	HT, hypercholesterolemia; female 56%; mean age 58.0 y.o; TC 6.5-10.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, PC (P)	Change in plasma lipids, lipoproteins and apolipoproteins	Prav (H)	10, 20 or 40	2.5, 5 or 10	0.5
LIPID[79]	Acute MI or diagnosed unstable angina (3-36 months before study entry), hypercholesterolemia; female 17%; mean age 62.0 y.o; TC 4.0-7.0, TG < 5.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Death from CHD	Prav (H)	40	10	6.1
LIPS[80]	Successful first PCI or $\geq$ 1 lesion in native coronary arteries, stable/unstable angina or silent ischemia; female 16%; mean age 60 y.o; TC 3.5-7.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Development of major adverse cardiac event	Fluv (L)	80	10	3.9
LISA[81]	Stable symptomatic CHD, hyperlipidaemia; female 38%; mean age 60.0 y.o; TC $\geq$ 6.5, LDL-C > 4.1, TG $\leq$ 3.4 (mmol L^{-1}); excluded if had history of intolerance to statins	R, DB, PC (P)	Incidence of cardiac event	Fluv (L)	40 or 80	5 to 10	1

LRT[82]	≥ 1 area of stenosis (50-99%) > 1.5 mm in vessel supplying viable myocardium, ≥ 90% chance of successful angioplasty; female 58%; mean age 62.0 y.o; TC 4.1-7.8 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	Pr, R, DB, PC (P)	Restenosis of index lesion	Lov (L)	80	20	0.5
Lye et al.† [83]	Elderly, hypercholesterolemia; female 60%; mean age 69.5; LDL-C ≥ 4.1 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Fluv (L)	40	5	0.23
Lynch et al.[84]	Aneurysmal subarachnoid haemorrhage within 48 hours before randomisation; female 85%; mean age 56.0 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in cerebral vasospasm	Simv (L)	80	40	0.04
MAAS[85]	CHD, undergoing routine coronary angiography; female 55%; mean age 88.5 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Diffuse coronary atherosclerosis and focal coronary atherosclerosis	Simv (L)	20	10	4
MARS[86]	CAD; female 9%; mean age 58.0 y.o; TC 4.9-7.6 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in diameter stenosis	Lov (L)	80	20	2
MEGA[87]	Hypercholesterolemia, no history of CHD or stroke; female 69%; mean age 58.3 y.o; TC 5.7-7.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	PROBE (P)	First occurrence of CHD	Prav (H)	10 or 20	2.5 or 5	5.3
METEOR[88]	10-y FRS of < 10%, CIMT measurements 1.2-3.5 mm, moderately elevated cholesterol; female 69%; mean age 58.3 y.o; LDL-C 3.1-4.9, HDL-C ≤ 1.6, TG < 5.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in maximum CIMT	Rosuv (H)	40	160	2

Meyers et al.[89]	Hypercholesterolemia, concurrent CAD or ≥ 2 other coronary risk factors; female 100%; mean age 48.8 y.o; LDL-C 4.1-4.9 (mmol L^{-1}); excluded if history of musculoskeletal diseases or elevated CK	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	20	5	0.23
MIRACL[90]	Chest pain/discomfort ≥ 15 mins at rest or minimal exertion within the 24 hours preceding hospitalisation; female 35%; mean age 65.0 y.o; TC < 7.0 (mmol L^{-1}); excluded if taking drugs associated with rhabdomyolysis with statins	R, DB, PC (P)	Death, nonfatal acute MI, cardiac arrest with resuscitation or recurrent symptomatic myocardial ischemia	Ator (L)	80	80	0.31
Mohebbi et al.[91]	Diagnosis of glial tumour undergoing elective surgery; female 40%; mean age 47.0 y.o; excluded if history of serious adverse reactions with atorvastatin	R, DB, PC (P)	Change in metalloproteinase-9	Ator (L)	80	80	0.06
Morgan et al.[92]	HT, hypercholesterolemia; female 4%; mean age 67.6 y.o; TC 5.5-7.5 (mmol L^{-1}), TC/HDL-C ratio > 4.5; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC and TC/HDL-C ratio	Simv (L)	10, 20 or 40	5, 10 or 20	0.35
MRC/BHF HPS[93]	Substantial 5 y risk of death from CHD; female 25%; mean age NR; TC ≥ 3.5 (mmol L^{-1}); excluded if history of inflammatory muscle disease, muscle problems or CK > 3xULN	R, DB, PC (P)	Death from all causes or CHD	Simv (L)	40	20	5
Napoli et al.[94]	Combined hyperlipidemia, history of CAD, endinous xanthoma/xanthelasma/corneal arcus; female 39%; mean age 49.0 y.o; TC > 7.2 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, PC, UC (P)	Change in LDL-C, HDL-C, TG and VLDL-C	Prav (H)	20	5	2
NPSG[95]	Primary hypercholesterolemia, ≥ 2 CHD risk factors; female 18%; mean age 54.2 y.o; TC 5.2-7.8 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG and	Prav (H)	20 or 40	5 or 10	0.5

			incidence of adverse events				
OCS[96]	Higher than average risk of CHD because of history of MI, AP, stroke, TIA, PVD, DM or HT; female 15%; mean age 63.5 y.o; TC > 3.5 (mmol L^{-1}); excluded if reported adverse events during run-in period	R, DB, PC (P)	Change in lipids, biochemistry and haematology measures	Simv (L)	20 or 40	10 or 20	3.4
PACT[97]	Unstable angina, non-ST-segment elevation MI or ST-segment elevation MI; female 24%, mean age 61.1 y.o; excluded if history of serious adverse reactions to statins	R, DB, PC (P)	Death, recurrence of MI or readmission to hospital for unstable angina	Prav (H)	20 or 40	5 or 10	0.08
Päivä et al.[98]	Hypercholesterolemia; female 31%; mean age 50 y.o; TC < 7.0 mmol L^{-1}; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in plasma sterol, muscle ubiquinone and respiratory chain enzyme activities	Ator (L) or Simv (L)	40 or 80	40	0.15
Panahi et al.[99]	Mild-to-moderate aortic stenosis; female 39%; mean age 69.5 y.o; no explicit myopathy/CK exclusion criteria	Pr, R, DB, PC (P)	Change in serum lipid profile, C-reactive protein and echocardiographic parameters	Ator (L)	20	20	1
Panichi et al.[100]	CRF, pre-dialysis, hypercholesterolemia; female 20%; mean age 67.5 y.o; LDL-C > 2.6 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in lipids, inflammatory markers and renal function	Simv (L)	40	20	0.5
Parker et al.† [101,102]	History of muscle problems with statins; female NR; mean age NR; only excluded if had history of severe rhabdomyolysis (CK > 10 x ULN)	R, DB, PC (CO)	Confirm statin-associated myalgia	Simv (L)	20	10	0.15

PEARL[103]	CHF, mild hypercholesterolemia; female 53%; mean age 62.6 y.o; TC ≤ 6.5, LDL-C ≤ 4.4 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	PROBE (P)	Cardiac death and hospitalisation for worsening HF	Pitv (L)	2	10	2.96
PLAC I[104]	CAD, mild-to-moderate hyperlipidemia; female 23%, mean age 57.0 y.o; LDL-C 3.4-4.9, TG < 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Fatal or nonfatal MI, CHD death, nonfatal infarction or death from any cause and total clinical events	Prav (H)	40	10	2.3
PMSGCRP[105]	Hypercholesterolemia, ≥ 2 additional CAD risk factors; female 24%; mean age 55.0 y.o; TC 5.2-7.8 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	20	5	0.5
PMSGD[106]	T2DM, hyperlipidaemia; female 49%; mean age 58.3 y.o; TC 5.2-7.8 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	10	2.5	0.31
Pollo-Flores et al.[107]	Cirrhosis with portal HT; female 47%; mean age 57.5 y.o; no explicit myopathy/CK exclusion criteria	Pr, R, TB, PC (P)	Change in hepatic venous pressure gradient	Simv (L)	40	20	0.25
PROCAS[108]	Congenital aortic stenosis; female 72%; mean age 32.5 y.o; excluded if history of muscle disease	R, DB, PC (P)	Change in peak aortic valve velocity	Rosuv (H)	10	40	2.4
PROSPER[109]	Pre-existing or high risk of vascular disease or stroke; female 52%; mean age 75.4 y.o; TC 4.0–9.0, TG < 6.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Definite or suspect death from CHD, non-fatal MI and fatal or non-fatal stroke	Prav (H)	40	10	3.2

PTT[110]	Undergone coronary balloon angioplasty of infarcted artery during 1st month of acute MI; female 17%; mean age 51.5 y.o; TC < 7.76; TG < 3.39 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Major adverse cardiovascular events and evidence of restenosis	Prav (H)	40	10	0.5
Raskin et al.[111]	T1DM/T2DM, hypercholesterolemia; female 53%; mean age 54.2 y.o; LDL-C > 3.9 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C and TG	Prav (H)	20	5	0.31
REGRESS[112]	$\geq$ 1 coronary stenosis (≥50%) in a major coronary artery, normal to moderately elevated TC; female 0%; mean age 56.2 y.o; TC 4.0-8.0 (mmol L^{-1}); excluded if history of muscle disorders	R, DB, PC (P)	Changes in MSD and MOD	Prav (H)	40	10	2
RIGHT[113]	Mixed hyperlipidaemia; female 37%; mean age 54.0 y.o; LDL-C $\geq$ 4.1, TG 5.2-13.0 (mmol L^{-1}); excluded if history of muscle disease	R, DB, PC (P)	Change in LDL-C	Ceriv (L)	0.1, 0.2 or 0.3	2.5, 5 or 7.5	0.31
ROSUV† [114]	Hypercholesterolemia; female 39%; mean age 56.0 y.o; LDL-C 4.1-5.6, TG < 3.4 (mmol L^{-1}); excluded if CK > 3xULN at screening	DB, DR, PC (P)	Change in LDL-C	Ator (L) or Rosuv (H)	Rosuv : 1, 2.5, 5, 10, 20 or 40 Ator: 10 or 80	4, 10, 20, 40, 80 or 160	0.12
ROSUVATOR[115]	Hypercholesterolemia; female 54%; mean age 57.0 y.o; LDL-C 4.1-6.5, TG < 4.5 (mmol L^{-1}); excluded if CK > 3xULN at screening	R, DB, PC (P)	Change in LDL-C	Ator (L) or	Rosuv : 5 or 10;	10, 20 or 40	0.23

				Rosuv (H)	Atorv: 10		
Saito et al.[116]	Hypercholesterolemia; female 30%; mean age 52.4 y.o; TG 2.3-5.7 (mmol L^{-1}); excluded if CK > 3xULN at screening	R, DB, PC (P)	Change in TG	Rosuv (H)	5, 10 or 20	20, 40 or 80	0.15
SALTIRE[117]	Calcific aortic stenosis, aortic-jet velocity $\geq$ 2.5 m/s, aortic-valve calcification; female 70%; mean age 68 y.o; TC < 4.0 (mmol L^{-1}); excluded if history of intolerance to statins	R, DB, PC (P)	Change in stenosis and valvular calcification	Ator (L)	80	80	2.1
Sano et al.[118]	Mild-to-moderate Alzheimer's disease; female 59%; mean age 74.6; LDL-C > 2.1, TG < 5.7 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in cognitive portion of the Alzheimer's Disease Assessment Scale score	Simv (L)	40	20	1.5
Santinga et al.[119]	Elderly, primary hypercholesterolemia; female 67%; mean age 67.0 y.o; LDL-C > 4.3, TG < 2.8 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	20	5	1.85
SHARP[120]	Advanced CKD, no known history of MI or coronary revascularisation; female 37%; mean age 60.8 y.o; excluded if history of active inflammatory muscle disease or CK > 3xULN	R, DB, PC (P)	Major atherosclerotic events	Simv (L)	20	10	2.5
Simsek et al.[121]	With or without erectile dysfunction; female 0%; mean age 58.6 y.o; no explicit myopathy/CK exclusion criteria	R, DB, UC (P)	Change in brachial artery flow-mediated dilatation and cavernosal arteries diameter	Ator (L)	10	10	0.08
SPARCL[122]	Stroke or TIA 1-6 months before study entry, no known CHD; female 40%; mean age 62.8 y.o; LDL-C 2.6-4.9 (mmol L^{-1}); excluded if CK > 5	R, DB, PC (P)	First nonfatal or fatal stroke	Ator (L)	80	80	4.9

	x ULN or taking drugs associated with rhabdomyolysis when used with statins						
STATCOPE[123]	Moderate-to-severe chronic obstructive pulmonary disease and smoking history; female 44%; mean age 62.3 y.o; no explicit myopathy/CK exclusion criteria	Pr, R, PC (P)	Change in Exacerbation rate (i.e. exacerbations per person-year)	Simv (L)	40	20	1.76
Stegmayr et al.[124]	Stage 4 or 5 CKD; female 31%; mean age 68.7 y.o; exclude if history of adverse reactions to statins	Pr, R, PC (P)	All-cause mortality, non-lethal acute MI, CABG surgery or PTCA	Ator (L)	10	10	3
Stein et al.[125]	T2DM, mixed hyperlipidaemia; female 48%; mean age 53.0 y.o; LDL-C > 3.4, TG 3.9-7.9 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (CO)	Change in LDL-C	Simv (L)	40 or 80	20 or 40	0.12
STOMP[126]	Healthy, statin-naïve; female 51%; mean age 44.1 y.o; no limits of LDL-C; excluded if history of subjective muscle complaints or weakness	R, DB, PC (P)	Muscle symptoms, CK, exercise capacity and muscle strength	Ator (L)	80	80	0.5
Strey et al.[127]	Stable symptomatic HF; female NR; mean age NR; no explicit myopathy/CK exclusion criteria	R, DB, PC (CO)	Change in endothelium-dependent forearm resistance vessel function	Ator (L)	40	40	0.12
Taneva et al.[† 128]	Combined hyperlipidaemia; female 0%; mean age 55.0 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in fasting lipid levels and flow-mediated dilation of the brachial artery	Ator (L)	80	80	0.12
Udawat and Goyal[129]	T2DM; female 50%; mean age 52.9 y.o; LDL-C > 2.6, HDL-C < 1.7, TG > 2.3 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	Pr, O, R, UC (P)	Change in TC, LDL-C, HDL-C and TG	Simv (L)	10 or 20	5 or 10	0.23

UK-HARP-I[130]	CKD; female 31%; mean age 53.0 y.o; no upper limit on TC; excluded if history of inflammatory muscle disease or CK > 3 x ULN	R, PC (P)	Change in serum lipids, liver function tests and CK	Simv (L)	20	10	1
Urso et al.[131]	Healthy; female 100%; mean age 23.6 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in muscle histology and genetic expression	Ator (L)	80	80	0.08
Villegas-Rivera et al.[132]	T2DM diabetes, HbA1c < 12%; female 46%; mean age 54.4 y.o; excluded if history of adverse reactions to statins	R, DB, PC (P)	Change in lipid peroxidation and nitric oxide level in plasma	Rosuv (H)	20	80	0.31
Wang et al.[133]	Hypercholesterolemia; female 46%; mean age 66.1 y.o; LDL-C 4.1-6.5 (mmol L^{-1}); excluded if CK > 3xULN at screening	R, DB, PC (P)	Change in LDL-C	Ator (L)	10	10	0.15
Wiklund et al.[134]	Familial hypercholesterolemia; female 48%; mean age 50.9 y.o; no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in TC, LDL-C, HDL-C and TG	Prav (H)	20 or 40	5 or 10	0.23
Wiklund et al.2[135]	Primary hypercholesterolemia; female 37%; mean age 52.8 y.o; TC $\geq$ 6.0, TG $\leq$ 4.0 (mmol L^{-1}); no explicit myopathy/CK exclusion criteria	R, DB, PC (P)	Change in LDL-C, VLDL-C, TG, HDL-C and apolipoprotein A-I and B	Prav (H)	40	10	0.23
WOSCOPS[136]	No history of MI, no serious ECG abnormalities or arrhythmias, moderate hypercholesterolemia; female 0%; mean age 55.2 y.o; LDL-C 4.5-6.0 (mmol L^{-1}); excluded if CK > 360 U/L at screening	R, DB, PC (P)	First occurrence nonfatal MI, death from CHD or death from CHD or MI	Prav (H)	40	10	4.9

† Data taken from first phase of study only (when there was a placebo-controlled group); ‡ Data taken from Phase A only; *Data taken from Group B participants only; **Data combined for usual care and lifestyle intervention groups

Abbreviations: ACS, acute coronary syndrome; ALT, alanine aminotransferase; AP, angina pectoris; ARF, acute renal failure; AST, aspartate aminotransferase; Ator, atorvastatin; BGL, blood glucose level; BL, baseline; BMI, body mass index; CAB, coronary artery bypass; CABG, coronary artery bypass graft; CAD, coronary artery disease; Ceriv, Cerivastatin; CHD, coronary heart disease; CgHF, congestive heart failure; CHF, chronic heart failure; CIMT, carotid intima-media thickness; CK, creatine kinase; CKD, chronic kidney disease; CRF, chronic renal failure; CTEPH, chronic thromboembolic pulmonary hypertension; CVA, cerebrovascular accident; CVD, cardiovascular disease; DB, double-blind;

DR, dose response; ECG, Electrocardiogram; Fluv, Fluvastatin; FRS, Framingham risk score; GGT, Gamma-glutamyl transferase; H, hydrophilic; HbA1c, Haemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HF, heart failure; HT, hypertension; L, lipophilic; LDL-C, low-density lipoprotein cholesterol; Lov, Lovastatin; LVEF, left ventricular ejection fractions; m, month; MI, myocardial infarction; MLD, minimal lumen diameter; MOD, minimum obstruction diameter; MSD, mean segment diameter; O, Open-label; OT, Open trial; PAH, pulmonary arterial hypertension; PAPP-A/proMBP, Pregnancy-associated plasma protein-A proform of eosinophil major basic protein; PC, placebo-controlled; PCI, percutaneous coronary intervention; Pitv, pitavastatin; Pr, prospective; Prav, pravastatin; PROBE, prospective, randomised, open-label, blinded-endpoint comparative study; PTCA, percutaneous transluminal coronary angioplasty; PVD, peripheral vascular disease; R, randomised; Rosuv, rosuvastatin; Simv, simvastatin; TB, triple-blind; TC, total cholesterol; TG, triglycerides; TIA, transient ischaemic attack; T1DM, Type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; UC, usual care; ULN, Upper limit of normal; VLDL-C, Very low-density lipoprotein cholesterol; y, years, y.o, years old

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Table S6 Adverse skeletal muscle side effects in randomised controlled trials included in a meta-analysis of statin lipophilicity, dose and muscle symptoms

Study	Total participants analysed		Study definitions of adverse muscle symptoms	Participants with muscle pain/weakness		Participants with myalgia		Participants with myositis		Participants with asymptomatic CK elevation < 10 x ULN or unspecified elevation		Participants with asymptomatic CK elevation ≥ 10 x ULN		Participants with rhabdomyolysis		Participants with myopathy		Total cases of adverse muscle symptoms (excluding asymptomatic CK elevations)	
	Statin	Control		Statin	Control	Statin	Control	Statin	Control	Statin	Control	Statin	Control	Statin	Control	Statin	Control	Statin	Control
4-D[1]	619	636	NR	NR	NR	Joined with myopathy	Joined with myopathy	NR	NR	12	4	NR	NR	0	0	7	5	7	5
4S[2]	2221	2223	NR	530	520	82	72	NR	NR	NR	NR	6	1	See myopathy	See myopathy	1	0	613	592
A to Z[3]	2265	2232	Myopathy – CK > 10 x ULN with associated muscle symptoms; Rhabdomyolysis – myopathy with CK > 10 000 IU/L	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	0	1	0	1
ACAPS[4]	231	230	NR	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0

AFCAPS/ TexCAPS[5]	3304	3301	Myopathy – muscle symptoms accompanied with CK > 10 x ULN	NR	NR	10	10	NR	NR	NR	NR	21	21	1	2	0	0	11	12
ALLIANCE[6]	1217	1225	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	0	0	0	0	0	0
APATH[7]	112	108	NR	NR	NR	2	3	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	2	3
APOLLO[8]	97	46	NR	NR	NR	2	0	NR	NR	1	0	NR	NR	NR	NR	NR	NR	2	0
ASA-STAT[9]	32	33	Myopathy – CK > 10 x ULN	NR	NR	3	5	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	3	5
ASCOT-LLA[10]	5168	5137	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0	NR	NR	1	0
ASEPSIS[11]	49	51	NR	NR	NR	0	0	NR	NR	2	0	NR	NR	NR	NR	NR	NR	0	0
ASPEN[12]	1211	1199	NR	NR	NR	36	19	NR	NR	NR	NR	NR	NR	1	1	NR	NR	37	20
ASTRONOMER[13]	134	135	NR	1	2	NR	NR	NR	NR	NR	NR	1	2	0	0	NR	NR	1	2
AURORA[14]	1389	1384	Rhabdomyolysis – muscle symptoms with CK > 10 x ULN	See myopathy	See myopathy	NR	NR	NR	NR	10	9	NR	NR	3	2	310	343	313	345
AVERT[15]	164	177	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	NR	NR	NR	NR	0	0

Bak et al.[16]	106	109	NR	2	1	NR	NR	NR	NR	0	0	0	0	NR	NR	NR	NR	2	1
Bays et al.[17]	622	148	Myopathy – CK ≥ 10 x ULN associated with muscle symptoms	NR	NR	NR	NR	NR	NR	NR	NR	1	1	0	0	1	1	1	1
Bays et al.2[18]	31	31	Predefined super-category of AE terms likely to indicate muscle pathology, including myalgia, muscle pain, muscle cramps, myopathy, myositis, muscle aches, muscle weakness, fibromyalgia, and CK increase (>5 x ULN)	See myopathy	See myopathy	See myopathy	See myopathy	See myopathy	See myopathy	0	0	0	0	NR	NR	4	2	4	2
Beigel et al.[19]	38	39	NR	NR	NR	NR	NR	NR	NR	1	0	0	0	NR	NR	0	1	0	1
Betteridge et al.[20]	966	192	NR	NR	NR	17	2	NR	NR	10	3	2	1	NR	NR	NR	NR	17	2
Bone et al.[21]	485	119	NR	NR	NR	53	8	NR	NR	0	0	0	0	0	0	NR	NR	53	8

Bruckert et al.[22]	607	622	NR	0	7	NR	NR	NR	NR	0	0	0	0	NR	NR	NR	NR	0	7
CARDS[23]	1428	1410	NR	NR	NR	61	72	NR	NR	NR	NR	2	10	0	0	1	1	62	73
CARE[24]	2081	2078	NR	NR	NR	NR	NR	0	4	12	7	NR	NR	NR	NR	NR	NR	0	4
Carlsson et al.[25]	29	28	NR	NR	NR	1	1	NR	NR	0	0	0	0	NR	NR	NR	NR	1	1
Cash et al.[26]	11	10	NR	NR	NR	1	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0
Chan et al.1[27]	30	30	Myopathy – CK elevation with muscle pain	0	1	NR	NR	NR	NR	1	1	NR	NR	NR	NR	1	0	1	1
Chan et al.2[28]	25	25	NR	2	2	NR	NR	NR	NR	0	0	0	0	NR	NR	NR	NR	2	2
Chan et al.3[29]	48	48	NR	NR	NR	1	2	NR	NR	4	3	0	0	NR	NR	NR	NR	1	2
Chan et al.4[30]	38	38	NR	See myalgia	See myalgia	1	1	NR	NR	3	3	0	0	NR	NR	NR	NR	1	1
Chua et al.[31]	6	8	Myalgia – mild muscle aches	NR	NR	1	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0
Cojocaru et al.[32]	50	50	NR	1	2	2	3	NR	NR	1	2	0	0	NR	NR	NR	NR	3	5

COMETS[33]	317	79	Myopathy – myalgia with CK elevation > 10 x ULN	4	0	5	2	NR	NR	0	0	0	0	0	0	0	0	9	2
CORONA[34]	2514	2497	Definitions as per Medical dictionary for Regulatory activities	See myopathy	See myopathy	See myopathy	See myopathy	See myopathy	See myopathy	NR	NR	1	3	NR	NR	225	207	225	207
Cowan et al.[35]	43	43	NR	1	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0
CRISP[36]	289	142	NR	6	0	NR	NR	NR	NR	0	0	0	0	NR	NR	0	0	95	47
CSG[37]	971	199	NR	NR	NR	26	7	NR	NR	14	2	12	0	NR	NR	9	0	35	7
Cubeddu et al.[38]	25	24	NR	NR	NR	0	0	0	0	1	0	0	0	NR	NR	NR	NR	0	0
Dadkhah et al.[39]	66	65	NR	NR	NR	2	0	NR	NR	0	0	0	0	NR	NR	NR	NR	2	0
Davidson et al.[40]	26	26	NR	NR	NR	3	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	3	0
DECREASE-IV[41]	265	268	Myopathy – CK elevation with/without muscle complaints; Rhabdomyolysis – CK > 10xULN with elevated creatinine	NR	NR	NR	NR	NR	NR	NR	NR	10	8	0	0	0	0	0	0

Dhamija et al.[42]	32	32	NR	4	3	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	4	3
DIATOR[43]	46	45	NR	NR	NR	1	0	NR	NR	16	6	NR	NR	NR	NR	NR	NR	1	0
Durazzo et al.[44]	50	50	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0	NR	NR	1	0
ESG[45]	263	70	Myopathy – CK > 10 x ULN with associated muscle symptoms	See myalgia	See myalgia	9	3	NR	NR	0	0	0	0	1	0	1	0	10	3
ESG2[46]	220	64	NR	10	2	NR	NR	NR	NR	NR	NR	0	0	0	0	0	0	10	2
ESG3[47]	205	65	Myopathy – CK > 10 x ULN with associated muscle symptoms	See myalgia	See myalgia	0	0	NR	NR	1	0	2	0	0	0	0	0	0	0
ESP[48]	258	89	NR	NR	NR	NR	NR	NR	NR	0	0	0	0	NR	NR	NR	NR	0	0
EXCEL[49]	6582	1663	Myopathy – CK > 10 x ULN with associated muscle symptoms	See myalgia	See myalgia	514	125	136	27	2060	481	NR	NR	0	0	5	0	655	152
EZET-ATOR[50]	248	60	Myopathy – CK > 10 x ULN with associated muscle symptoms	NR	NR	0	0	0	0	NR	NR	0	0	NR	NR	14	3	14	3
FACS[51]	78	78	NR	NR	NR	NR	NR	NR	NR	0	0	0	0	NR	NR	0	0	0	0

FLARE[52]	526	528	Myopathy – CK > 10 x ULN with muscle symptoms	NR	NR	9	3	NR	NR	NR	NR	0	0	NR	NR	0	0	9	3
Fogari et al.[53]	16	16	NR	NR	NR	1	0	NR	NR	0	0	0	0	NR	NR	NR	NR	1	0
GAUSS-3[54]	491	492	NR	See myopathy	See myopathy	See myopathy	See myopathy	See myopathy	See myopathy	NR	NR	NR	NR	See myopathy	See myopathy	209	130	209	130
Gentile et al.[55]	323	86	NR	NR	NR	2	0	0	0	0	0	0	0	0	NR	NR	0	0	2	0
Ghirlanda et al.[56]	20	10	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	0	0
GISSI-P[57]	2138	2133	NR	6	0	NR	NR	NR	NR	NR	NR	NR	NR	0	0	NR	NR	6	0
GISSI-HF[58]	2285	2289	NR	23	21	NR	NR	NR	NR	9	2	1	1	0	0	NR	NR	23	21
GREACE[59]	800	800	Myalgia – muscle symptoms without elevation in CK; Myopathy – myalgia with CK 5-10 x ULN	NR	NR	0	0	NR	NR	0	0	0	0	NR	NR	0	0	0	0

Hommel et al.[60]	12	9	NR	NR	NR	1	0	NR	NR	0	0	0	0	NR	NR	NR	NR	1	0
HOPE-3[61]	6361	6344	NR	367	296	NR	NR	NR	NR	NR	NR	NR	NR	See myopathy	See myopathy	2	1	369	297
Hunningh ake et al.[62]	180	88	NR	NR	NR	4	2	NR	NR	6	3	0	0	NR	NR	NR	NR	4	2
Hunningh ake et al.2[63]	130	26	Myopathy – muscle symptoms and CK > 10 x ULN	NR	NR	7	0	NR	NR	NR	NR	1	0	NR	NR	2	0	9	0
HYRIM[64]	283	285	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	1	0	0	NR	NR	0	0
Jacobson et al.[65]	182	63	NR	15	4	NR	NR	NR	NR	0	0	0	0	NR	NR	1	0	16	4
John et al.[66]	33	37	NR	7	2	NR	NR	NR	NR	0	1	NR	NR	NR	NR	NR	NR	7	2
JUPITER[67]	8901	8901	NR	1421	1375	NR	NR	NR	NR	NR	NR	NR	NR	1	0	10	9	1432	1384
KAPS[68]	224	223	NR	51	45	NR	NR	NR	NR	9	11	0	0	NR	NR	0	0	51	45
Kennedy et al.[69]	15	17	NR	NR	NR	3	2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	3	2
Kondurac ka et al.[70]	154	50	Myopathy – CK > 10 x ULN	NR	NR	2	0	NR	NR	4	0	NR	NR	NR	NR	NR	NR	2	0

Krysiak & Okopień[71]	23	23	Myopathy – CK > 10 x ULN	NR	NR	1	0	NR	NR	0	0	0	0	NR	NR	NR	NR	1	0
Krysiak et al.[72]	49	47	Myopathy – CK > 10 x ULN	NR	NR	NR	NR	NR	NR	1	0	0	0	NR	NR	0	0	0	0
Krysiak et al.2[73]	25	24	Myopathy – CK > 10 x ULN	NR	NR	1	0	NR	NR	0	0	0	0	NR	NR	NR	NR	1	0
Krysiak et al.3[74]	46	42	NR	NR	NR	2	0	NR	NR	0	0	0	0	NR	NR	NR	NR	2	0
Krysiak et al.4[75]	24	22	NR	NR	NR	1	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0
LCAS[76]	214	215	NR	NR	NR	NR	NR	NR	NR	1	2	NR	NR	NR	NR	0	0	0	0
Lewis et al.[77]	163	163	NR	NR	NR	2	0	NR	NR	NR	NR	NR	NR	0	1	NR	NR	2	1
Lijnen et al.[78]	25	25	NR	4	1	NR	NR	NR	NR	6	5	NR	NR	NR	NR	0	1	4	2
LIPID[79]	4512	4502	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	8	10	8	10
LIPS[80]	844	833	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	3	0	0	NR	NR	0	0
LISA[81]	187	178	NR	NR	NR	NR	NR	NR	NR	0	1	NR	NR	NR	NR	NR	NR	0	0
LRT[82]	203	201	NR	NR	NR	NR	NR	NR	NR	NR	NR	3	0	NR	NR	NR	NR	0	0
Lye et al.[83]	33	36	NR	2	1	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	2	1

Lynch et al.[84]	19	20	Myositis – CK > 1000 U/L	NR	NR	NR	NR	0	0	NR	NR	1	0	NR	NR	0	0	0	0
MAAS[85]	193	188	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	0	0
MARS[86]	123	124	NR	NR	NR	2	0	NR	NR	NR	NR	NR	NR	NR	NR	0	0	2	0
MEGA[87]	3866	3966	NR	NR	NR	NR	NR	NR	NR	111	98	NR	NR	0	0	NR	NR	0	0
METEOR[88]	700	281	Rhabdomyolysis – muscle symptoms with CK > 10 x ULN, creatinine elevation, brown urine and urinary myoglobin	13	13	89	34	NR	NR	17	0	1	2	0	0	NR	NR	102	47
Meyers et al.[89]	172	58	NR	18	8	9	4	0	0	0	1	NR	NR	NR	NR	NR	NR	27	12
MIRACL[90]	1538	1548	NR	NR	NR	NR	NR	0	0	NR	NR	NR	NR	0	0	NR	NR	0	0
Mohebbi et al.[91]	21	21	NR	0	0	0	0	NR	NR	NR	NR	NR	NR	NR	NR	0	0	0	0
Morgan et al.[92]	24	25	NR	2	3	NR	NR	NR	NR	5	4	NR	NR	NR	NR	NR	NR	2	3
MRC/BHF HPS[93]	10269	10267	Myopathy – muscle symptoms with CK> 10 x ULN; Rhabdomyolysis –	3379	3409	NR	NR	NR	NR	19	13	11	6	5	3	5	1	3389	3413

			muscle symptoms with CK > 40 x ULN																
Napoli et al.[94]	13	14	Myopathy – CK > 10 x ULN with myalgia	See myalgia	See myalgia	1	0	NR	NR	1	0	0	0	0	0	0	0	1	0
NPSG[95]	79	75	NR	6	5	NR	NR	NR	NR	15	14	0	0	NR	NR	NR	NR	6	5
OCS[96]	414	207	NR	225	106	6	2	NR	NR	16	8	0	0	NR	NR	4	2	229	114
PACT[97]	1710	1698	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	NR	NR	0	0	0	0
Päivä et al.[98]	32	16	NR	0	0	NR	NR	NR	NR	0	0	0	0	NR	NR	NR	NR	0	0
Panahi et al.[99]	38	37	NR	NR	NR	NR	NR	NR	NR	2	0	NR	NR	NR	NR	0	0	0	0
Panichi et al.[100]	28	27	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0	1	0
Parker et al.[101,102]	135	135	NR	See myalgia	See myalgia	43	35	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	43	35
PEARL[103]	288	286	NR	NR	NR	4	1	NR	NR	3	0	NR	NR	NR	NR	NR	NR	4	1
PLAC I[104]	206	202	Myopathy – myalgia with CK > 10 x ULN	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0	0	0

PMSGCRP[105]	530	532	Myopathy – myalgia associated with CK > 10 x ULN	2	3	See muscle pain/weaknes s	See muscle pain/weaknes s	NR	NR	14	8	0	0	NR	NR	0	0	2	3
PMSGD[106]	167	158	Myopathy – myalgia with CK > 10 x ULN	2	1	0	2	NR	NR	1	3	0	0	NR	NR	0	0	2	3
Pollo-Flores et al.[107]	14	20	NR	NR	NR	1	2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	2
PROCAS[108]	30	33	NR	3	1	NR	NR	NR	NR	5	4	0	0	0	0	NR	NR	3	1
PROSPER[109]	2891	2913	NR	NR	NR	36	32	NR	NR	NR	NR	0	0	0	0	NR	NR	36	32
PTT[110]	37	40	Myopathy – myalgia and CK > 10 x ULN	NR	NR	NR	NR	NR	NR	0	0	0	0	NR	NR	0	0	0	0
Raskin et al.[111]	62	32	Myopathy – myalgia with CK > 10 x ULN	8	3	NR	NR	NR	NR	0	0	0	0	NR	NR	0	0	8	3
REGRESS[112]	450	434	NR	NR	NR	1	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	0
RIGHT[113]	412	59	NR	NR	NR	3	2	NR	NR	12	0	NR	NR	NR	NR	NR	NR	3	2
ROSUV[114]	175	31	NR	NR	NR	6	1	NR	NR	0	0	0	0	NR	NR	0	0	6	1

ROSUVATOR[115]	387	132	NR	NR	NR	7	0	NR	NR	0	0	0	0	NR	NR	NR	NR	7	0
Saito et al.[116]	92	35	NR	NR	NR	4	1	NR	NR	0	0	0	0	0	0	0	0	4	1
SALTIRE[117]	77	78	NR	NR	NR	NR	NR	0	0	3	0	0	0	0	0	NR	NR	0	0
Sano et al.[118]	204	202	NR	NR	NR	11	10	NR	NR	0	0	0	0	NR	NR	NR	NR	11	10
Santinga et al.[119]	94	48	Myopathy – myalgia CK > 10 x ULN	42	19	NR	NR	NR	NR	0	0	0	0	NR	NR	0	0	42	19
SHARP[120]	1054	4191	Myopathy – CK > 10 x ULN with muscle symptoms or organ damage; Rhabdomyolysis – myopathy with CK > 40 x ULN with end-organ damage	103	393	1	3	NR	NR	5	11	1	1	0	1	0	4	104	401
Simsek et al.[121]	50	50	NR	0	0	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0	0
SPARCL[122]	2365	2366	NR	NR	NR	129	141	NR	NR	NR	NR	2	0	2	3	7	7	138	151

STATCOPE[123]	433	452	NR	NR	NR	See myopathy	See myopathy	NR	NR	NR	NR	NR	NR	NR	NR	10	11	10	11
Stegmayr et al.[124]	70	73	NR	NS	NS	NR	NR	NR	NR	0	0	0	0	0	0	NR	NR	0	0
Stein et al.[125]	260	130	NR	NR	NR	4	1	NR	NR	NR	NR	NR	NR	NR	NR	0	0	4	1
STOMP[126]	203	217	Statin-induced myalgia – new or increased muscle cramps not associated with exercise, persisted for $\geq$ 2 weeks, resolved within 2 weeks of stopping statin and reoccurred within 4 weeks of restarting medication	23	14	19	10	NR	NR	40	29	0	0	NR	NR	0	0	42	24
Strey et al.[127]	24	24	NR	NR	NR	1	0	NR	NR	0	0	0	0	NR	NR	NR	NR	1	0
Taneva et al.[128]	23	10	NR	NR	NR	1	0	NR	NR	1	0	NR	NR	NR	NR	NR	NR	1	0
Udawat and Goyal[129]	40	40	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	1	1	1	1

UK-HARP-I[130]	224	224	Myopathy – CK > 10 x ULN with muscle symptoms	44	37	NR	NR	NR	NR	1	3	1	0	0	0	0	0	44	37
Urso et al.[131]	4	4	NR	NR	NR	0	0	NR	NR	0	0	0	0	NR	NR	0	0	0	0
Villegas-Rivera et al.[132]	25	24	NR	NR	NR	4	0	NR	NR	NR	NR	NR	NR	NR	NR	1	0	5	0
Wang et al.[133]	26	28	Myopathy – myalgia with CK > 10 x ULN	NR	NR	NR	NR	NR	NR	0	0	1	0	NR	NR	0	0	0	0
Wiklund et al.[134]	40	40	NR	5	2	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	5	2
Wiklund et al.2[135]	64	69	Myopathy – CK > 10 000 IU/L with muscle pain or weakness	1	3	NR	NR	NR	NR	1	1	0	0	0	0	0	0	1	3
WOSCOPS[136]	3302	3293	NR	97	102	20	19	NR	NR	NR	NR	3	1	NR	NR	NR	NR	117	121

Abbreviations: CK, creatine kinase; NR, results not reported; NS, Information on muscle-related adverse events could not be discerned from other adverse events data; ULN, upper limit of normal.

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Table S7 Results of RCTs included in a meta-analysis of statin lipophilicity, dose and muscle symptoms following exclusion of the GAUSS-3[†] trial and HOPE-3 study[‡]

	Excluding GAUSS-3	Excluding HOPE-3
	Risk ratio (95% confidence interval)	
	Risk of muscle symptoms	
Overall meta-analysis	1.020 (0.993, 1.048), *P* = 0.155	1.024 (0.996, 1.052), *P* = 0.089
Subgroup analysis:		
LDL-C entry criteria (mmol L^{-1}):		
≥ 3.4	1.083 (0.977, 1.200), *P* = 0.130, n = 30	1.083 (0.977, 1.200), *P* = 0.130, n = 30
< 3.4	1.035 (0.967, 1.108), *P* = 0.317, n = 2	1.035 (0.967, 1.108), *P* = 0.317, n = 2
< 3.4 and ≥ 3.4	1.063 (0.881, 1.284), *P* = 0.521, n = 16	1.244 (0.973, 1.591), *P* = 0.081, n = 17
Not specified	1.011 (0.980, 1.044), *P* = 0.479, n = 54	1.002 (0.970, 1.035), *P* = 0.912, n = 53
Myopathy/CK/statin sensitivity exclusion criteria:		
Explicit criteria	1.004 (0.974, 1.035), *P* = 0.797, n = 36	1.004 (0.974, 1.035), *P* = 0.797, n = 36
No explicit criteria	1.088 (1.024, 1.157), *P* = 0.007, n = 66	1.116 (1.048, 1.188), *P* = 0.001, n = 66
Lipophilicity:		
Lipophilic	1.012 (0.979, 1.045), *P* = 0.483, n = 59	1.101 (1.025, 1.183), *P* = 0.009, n = 60

Hydrophilic	1.039 (0.988, 1.091), *P* = 0.134, n = 39	1.016 (0.964, 1.071), *P* = 0.553, n = 38
Prescribed dose:		
Low (< 40 mg*)	1.008 (0.974, 1.043), *P* = 0.650, n = 55	1.106 (1.021, 1.199), *P* = 0.014, n = 56
High (≥ 40 mg*)	1.056 (0.958, 1.164), *P* = 0.275, n = 18	1.015 (0.928, 1.110), *P* = 0.748, n = 17
Lipophilicity and dose treatment combination:		
Lipophilic+Low	1.009 (0.976, 1.043), *P* = 0.593, n = 45	1.096 (1.014, 1.185), *P* = 0.021, n = 46
Lipophilic+High	1.345 (0.887, 2.040), *P* = 0.163, n = 9	1.345 (0.887, 2.040), *P* = 0.163, n = 9
Hydrophilic+Low	1.050 (0.904, 1.221), *P* = 0.521, n = 27	1.050 (0.904, 1.221), *P* = 0.521, n = 27
Hydrophilic+High	1.041 (0.938, 1.155), *P* = 0.454, n = 9	0.999 (0.918, 1.088), *P* = 0.988, n = 8
Median follow-up period:		
≥ 6 m	1.017 (0.990, 1.045), *P* = 0.229, n = 56	1.010 (0.982, 1.038), *P* = 0.486, n = 55
< 6 m	1.209 (0.981, 1.490), *P* = 0.075, n = 46	1.426 (1.244, 1.634), *P* = < 0.001, n = 47
Mean participant age:		
≥ 65 y.o	1.072 (1.017, 1.130), *P* = 0.010, n = 22	1.050 (0.992, 1.111), *P* = 0.090, n = 21
< 65 y.o	1.016 (0.962, 1.073), *P* = 0.579, n = 76	1.076 (1.002, 1.156), *P* 0.044, n = 77
Gender:		
Only female participants	1.085 (0.515, 2.285), *P* = 0.831, n = 2	1.085 (0.515, 2.285), *P* = 0.831, n = 2
Only male participants	1.041 (0.852, 1.273), *P* = 0.693, n = 7	1.041 (0.852, 1.273), *P* = 0.693, n = 7

[†]Nissen SE, Stroes E, Dent-Acosta RE, et al. Efficacy and tolerability of evolocumab vs ezetimibe in patients with muscle-related statin intolerance: The GAUSS-3 randomized clinical trial. JAMA 2016;315:1580-90; [‡]Yusuf S, Bosch J, Dagenais G, et al. Cholesterol lowering in intermediate-risk persons without cardiovascular disease. N Engl J Med 2016;374:2021-31; *All doses normalised to atorvastatin dose equivalents; CK, creatine kinase; LDL-C, low-density lipoprotein cholesterol; m, months; RCTs, randomised controlled trial; y.o, years old

Table S8 Results of RCTs included in a meta-analysis of statin lipophilicity, dose and muscle symptoms following exclusion of the GAUSS-3 trial or winsorisation[†]

	Excluding GAUSS-3	All studies (Non-winsorised)	Winsorised results
	Risk ratio (95% confidence interval)		
	Risk of muscle symptoms		
Overall meta-analysis	1.020 (0.993, 1.048), P = 0.155, I^2 = < 0.001%	1.050 (1.014, 1.089), P = 0.007; I^2 = 3.291%	1.021 (0.994, 1.049), P = 0.135, I^2 = < 0.001%
Subgroup analysis:			
LDL-C entry criteria (mmol L^{-1}):			
≥ 3.4	1.083 (0.977, 1.200), P = 0.130, n = 30	1.083 (0.977, 1.200), P = 0.130, n = 30	1.083 (0.977, 1.200), P 0.130, n = 30
< 3.4	1.035 (0.967, 1.108), P = 0.317, n = 2	1.035 (0.967, 1.108), P = 0.317, n = 2	1.035 (0.967, 1.108), P = 0.317, n = 2
< 3.4 and ≥ 3.4	1.063 (0.881, 1.284), P = 0.521, n = 16	1.244 (0.973, 1.591), P = 0.081, n = 17	1.077 (0.912, 1.274), P = 0.382, n = 17
Not specified	1.011 (0.980, 1.044), P = 0.479, n = 54	1.011 (0.980, 1.044), P = 0.479, n = 54	1.011 (0.980, 1.044), P = 0.479, n = 54
Myopathy/CK/statin sensitivity exclusion criteria:			
Explicit criteria	1.004 (0.974, 1.035), P = 0.797, n = 36	1.004 (0.974, 1.035), P = 0.797, n = 36	1.004 (0.974, 1.035), P = 0.797, n = 36
No explicit criteria	1.088 (1.024, 1.157), P = 0.007, n = 66	1.134 (1.070, 1.202), P = < 0.001, n = 67	1.092 (1.028, 1.160), P = 0.005, n = 67
Lipophilicity:			
Lipophilic	1.012 (0.979, 1.045), P = 0.483, n = 59	1.101 (1.025, 1.183), P = 0.009, n = 60	1.013 (0.981, 1.046), P = 0.429, n = 60
Hydrophilic	1.039 (0.988, 1.091), P = 0.134, n = 39	1.039 (0.988, 1.091), P = 0.134, n = 39	1.039 (0.988, 1.091), P = 0.134, n = 39

Prescribed dose:			
Low (< 40 mg*)	1.008 (0.974, 1.043), *P* = 0.650, n = 55	1.106 (1.021, 1.199), *P* = 0.014, n = 56	1.009 (0.976, 1.044), *P* = 0.583, n = 56
High (≥ 40 mg*)	1.056 (0.958, 1.164), *P* = 0.275, n = 18	1.056 (0.958, 1.164), *P* = 0.275, n = 18	1.056 (0.958, 1.164), *P* = 0.275, n = 18
Lipophilicity and dose treatment combination:			
Lipophilic+Low	1.009 (0.976, 1.043), *P* = 0.593, n = 45	1.096 (1.014, 1.185), *P* = 0.021, n = 46	1.010 (0.978, 1.044), *P* = 0.532, n = 46
Lipophilic+High	1.345 (0.887, 2.040), *P* = 0.163, n = 9	1.345 (0.887, 2.040), *P* = 0.163, n = 9	1.345 (0.887, 2.040), *P* = 0.163, n = 9
Hydrophilic+Low	1.050 (0.904, 1.221), *P* = 0.521, n = 27	1.050 (0.904, 1.221), *P* = 0.521, n = 27	1.050 (0.904, 1.221), *P* = 0.521, n = 27
Hydrophilic+High	1.041 (0.938, 1.155), *P* = 0.454, n = 9	1.041 (0.938, 1.155), *P* = 0.454, n = 9	1.041 (0.938, 1.155), *P* = 0.454, n = 9
Median follow-up period:			
≥ 6 m	1.017 (0.990, 1.045), *P* = 0.229, n = 56	1.017 (0.990, 1.045), *P* = 0.229, n = 56	1.017 (0.990, 1.045), *P* = 0.229, n = 56
< 6 m	1.209 (0.981, 1.490), *P* = 0.075, n = 46	1.426 (1.244, 1.634), *P* = < 0.001, n = 47	1.214 (1.011, 1.457), *P* = 0.038, n = 47
Mean participant age:			
≥ 65 y.o	1.072 (1.017, 1.130), *P* = 0.010, n = 22	1.072 (1.017, 1.130), *P* = 0.010, n = 22	1.072 (1.017, 1.130), *P* = 0.010, n = 22
< 65 y.o	1.016 (0.962, 1.073), *P* = 0.579, n = 76	1.076 (1.002, 1.156), *P* = 0.044, n = 77	1.020 (0.966, 1.076), *P* = 0.482, n = 77
Gender:			
Only female participants	1.085 (0.515, 2.285), *P* = 0.831, n = 2	1.085 (0.515, 2.285), *P* = 0.831, n = 2	1.085 (0.515, 2.285), *P* = 0.831, n = 2
Only male participants	1.041 (0.852, 1.273), *P* = 0.693, n = 7	1.041 (0.852, 1.273), *P* = 0.693, n = 7	1.041 (0.852, 1.273), *P* = 0.693, n = 7

[†] Data was winsorised by replacing the results of the GAUSS-3 trial with the closest results of a study with similar design and setting – Taylor BA, Panza G, Thompson PD. Increased creatine kinase with statin treatment may identify statin-associated muscle symptoms. Int J Cardiol 2016;209:12-3. *All doses normalised to atorvastatin dose equivalents; CK, creatine kinase; LDL-C, low-density lipoprotein cholesterol; m, months; RCTs, randomised controlled trial; y.o, years old

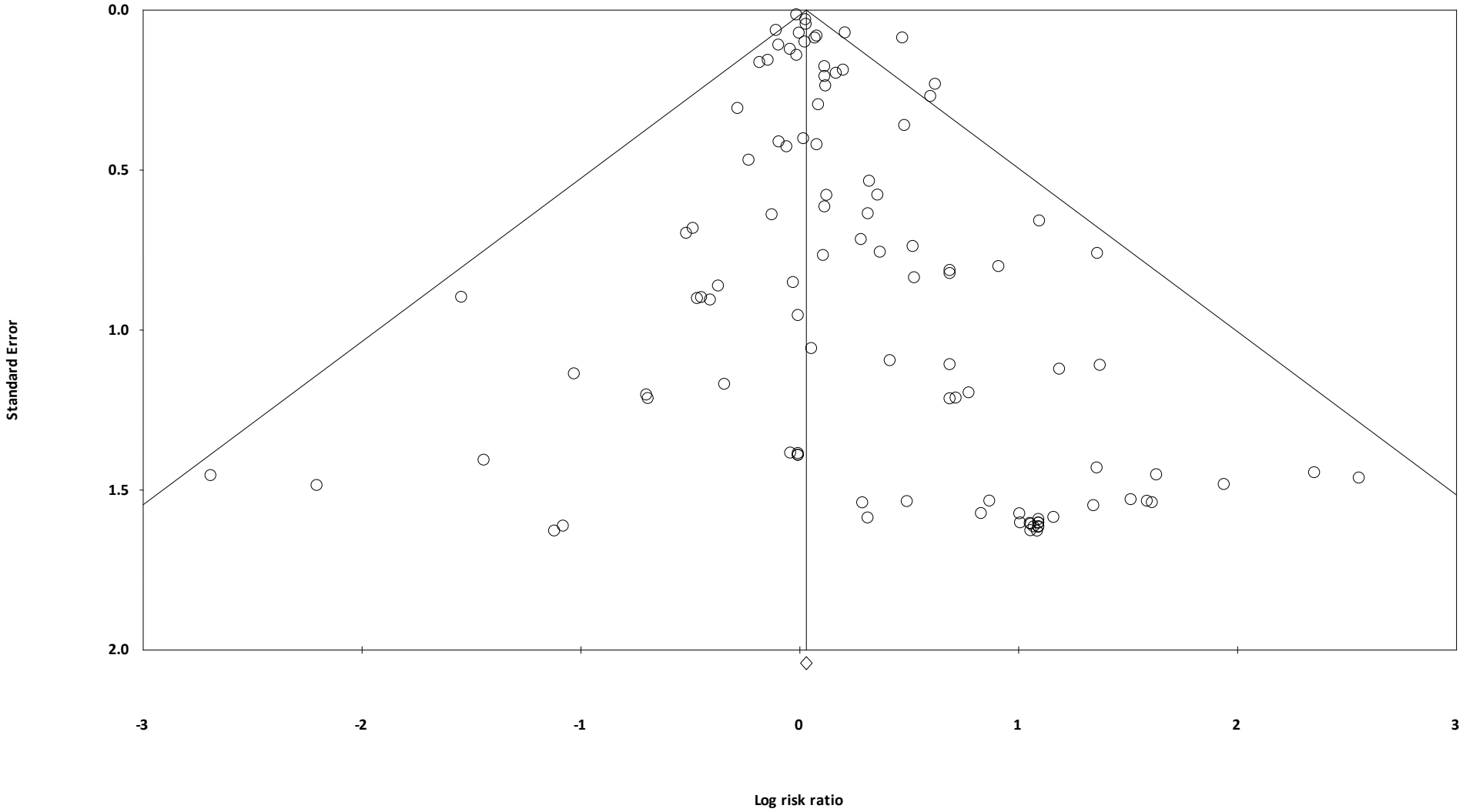

Fig. S1

APPENDIX B

General Methodology

Preamble

The methodology sections detailed in the chapters of this thesis were presented in a succinct manner in order to meet journal requirements for publication. This Appendix provides additional explanation of the experimental techniques used in this project. This information has been included as an Appendix, rather than a separate chapter, to avoid excessive repetition of ideas in this thesis.

B. General Methodology

B.1. Animals and administration of pharmaceuticals

B.1.1. Ethical approval

All experimental treatments and protocols employed in this thesis were approved by the Animal Ethics Committee of Central Queensland University (specific approval numbers are reported within each corresponding chapter). Rodents were housed on a constant 12-hour light-darkness cycle, and at temperature of 22 $\pm$ 2 °C, in order to stimulate a normal living environment. Access to water and food (standard rat chow) was provided *ad libitum*. The health of the rats was monitored during the treatment periods by measuring the mass of each animal every two days, as well as by assessing 24-hour food/water intakes three times per week.

B.1.2. Animals

Young female Wistar rats were used for all rodent-based studies in this project. Specifically, treatment was initiated when the rats were 12-weeks old (which approximates to an adult age in humans [1]. Although humans typically do not initiate statin treatment before middle-age [2] (the equivalent of 18 months in rodents), a younger animal model was needed in order to ensure that the effects of age-related sarcopenia (which is well-established in rats by 18 months of age [3]) would not influence the results of this study. Females were used because, compared to males, they are more sensitive to statin-induced myotoxicity and are thus considered to be the most appropriate sex for mechanistic studies of statin-associated muscle symptoms (SAMS) [4-6]. On this note, younger animals were also required in order to guarantee that

age-related loss of ovarian function would not influence the results obtained for the cardiac and vascular smooth muscle assessments [7].

B.1.3. Pharmaceuticals and treatment protocols

All pharmaceuticals were administered orally (i.e. via oral gavage) to replicate the route of administration of statins in humans. Pharmaceutical-grade statin tablets (simvastatin 80 mg or pravastatin 80 mg) were crushed (using a mortar and pestle) and dissolved in a 10% v/v solution of Polysorbate20 in milli-Q water. Polysorbate20 has been employed as both a control and a vehicle control in previous investigations [8-10]. Toxicological studies have also confirmed that oral administration of polysorbates provides no significant effects on body mass, food consumption or cardiovascular parameters [11]. Hence, this vehicle was suitable to use in this research project. Geranylgeraniol (GGOH) was dissolved in 0.9% saline solution as this is also an appropriate vehicle for administrating pharmaceutical compounds (particularly the small volumes used in this study) [12, 13]. This precursor of geranylgeranyl pyrophosphate (GGPP) was administered as it is more readily absorbed by cells/tissues [14, 15]. Therefore, it is a more appropriate formulation for observing the physiological effects of GGPP repletion.

B.2. Terminal experiments

B.2.1. Euthanasia of animals

Upon the completion of the treatment period, all animals were euthanised via a 1.0 mL intraperitoneal injection of sodium pentobarbital/pentobarbitone. This agent was diluted with milli-Q water (1:1 ratio) in order to obtain a concentration of 187.5 mg mL^{-1}. The absence of

brainstem reflexes (i.e. pedal and corneal reflexes) served as confirmation of death following the injection [16]. All terminal experiments/tissue collections were conducted during daylight hours (morning and afternoon) and animals were not fasted prior to euthanasia. Pilot studies had demonstrated that the SIM80 regimen could cause physiological distress. Hence, for ethical reasons, the rodents were not fasted prior to euthanasia in order to avoid worsening any potential distress.

B.2.2. Assessment of tissue/organ mass

Upon euthanasia, selected tissues/organs (including the left ventricle, kidneys, gastrocnemius, soleus and tibialis anterior) were isolated, blotted dry and weighed to obtain their wet mass. This assessment enabled evidence of atrophy/hypertrophy to be detected. Three different skeletal muscles of varying fibre-type composition were evaluated in this study as this allowed for the fibre-selective toxicity of statins to be evaluated.

B.2.3. Assessment of skeletal muscle function

Isolated skeletal muscle organ baths were completed using a methodology adapted from Simsek Ozek et al. [17]. Skeletal muscles were excised and placed in modified Krebs-Henseleit buffer (KHB) (KHB, all in mM concentrations: sodium chloride 119, potassium chloride 5, magnesium sulfate 1, potassium dihydrogen phosphate 1, sodium hydrogen carbonate 25, glucose 11 and calcium chloride 2; pH ~ 7.4). The tissues were tied with silk suture at their superior and inferior ends and mounted in vertical 25 mL organ baths containing warmed ($37^{o}C$), gassed KHB (oxygen (O_2) 95% / carbon dioxide (CO_2) 5%). The inferior end of each muscle was fastened to a glass hook (positioned between two platinum zigzag

electrodes) and the superior end was connected to an FT03 force displacement transducer (Grass Technologies, Middleton, WI, USA). Tissues were loaded with 2 g tension and then electrical field simulation trains (pulses 0.1 msec duration at 100 V) were applied for 5 seconds every 135 seconds at increasing frequencies of 1, 2, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Hz (stimulation trains were induced twice at each frequency). Changes in force were recorded using Lab Chart software and PowerLab® data acquisition units (ADInstruments, Bella Vista, NSW, Australia). Upon completion of the experiment, the muscles were weighed, and their lengths were measured. Force production was normalised by the muscle cross-sectional area according to the following equation: g/cm^2 = [force (g) × specific density of skeletal muscle (1.06 g/cm^3) × length of muscle (cm)] / [mass of muscle (g)] [17]. Non-linear regression was used to calculate $Log_{10}EF50$ values and statistical analyses were performed as described in section B.3.2.

B.2.4. Assessment of cardiac muscle function

The Langendorff heart preparation was used to evaluate the mechanical pumping function of the myocardium. This assessment was performed using a protocol adapted from Chan et al. [18]. Intact hearts were rapidly excised and submerged in ice-cold modified KHB. The aorta was cleaned of fat, cannulated and retrogradely perfused with warmed, gassed (O_2 95% / CO_2 5%) modified KHB at a constant pressure of 100 mmHg. Hearts were paced at 250 bpm by electrode stimulation of the right atrium for the entirety of the experiment. A latex balloon (filled with milli-Q water) was placed in the left ventricle and inflated at increasing increments of 5 mmHg every 60 seconds to generate a pressure-volume curve (range 0 mmHg to 30 mmHg). The balloon was connected to a Capto SP844 physiological pressure transducer (MLT 844/D) and responses were recorded using LabChart software. Using this information, the

diastolic stiffness of the myocardium was calculated according to the methodology described by Jackson et al. [19]. In short, the slope of the linear relationship between stress (σ, dyn/cm^2) and tangent elastic modulus (E, dyn/cm^2) was used to obtain the myocardial stiffness constant k (dimensionless). End systolic pressure, velocity of contraction/relaxation and developed pressure were measured at an intraventricular pressure of 10 mmHg. A timed collection of the coronary perfusate that dripped from the heart was used to determine the coronary flow (again, this measure was obtained at a pressure of 10 mmHg) [20]. Individual values were pooled to obtain a group value and inter-group differences were evaluated as per the details provided in section B.3.2.

B.2.5. Assessment of left ventricular electrophysiology

Single-cell microelectrode studies were performed in accordance with protocols detailed by Fenning et al. [21]. Following euthanasia, the least branching papillary muscle was dissected from the heart and transferred to a 1 mL experimental chamber perfused with warmed, gassed (O_2 95% / CO_2 5%) Tyrode's physiological salt solution (TPSS, all in mM concentrations: sodium chloride 137, potassium chloride 5, magnesium chloride 1, sodium dihydrogen phosphate 0.4, sodium bicarbonate 23, calcium chloride 2, glucose 6, ascorbic acid 0.3 and ethylenediaminetetraacetic acid 0.1; pH ~ 7.4). Tissues were fixed between two platinum electrodes and secured to a modified SensoNor AE 801 micro-force transducer (via the superior end) using a stainless-steel hook. The papillary muscles were gradually extended to obtain a maximum preload of 5 mN. Contractions at a frequency of 1 Hz, pulse width of 0.5 msec and stimulus strength 20% above threshold were subsequently induced using electrical field stimulation (Grass SD-9). Tissues were impaled with a glass electrode filled with 3 M potassium chloride (filamented borosilicate, outer diameter 1.5 mm, tip resistance 5–15 mΩ),

and any electrical activity was detected using a Cyto 721 electrometer (World Precision Instruments, Sarasota, Florida, United States). A minimum of three impalements were made per muscle (each at different locations) to obtain a total of 30 minutes of recording. Using this information, the following parameters were assessed: action potential durations, resting membrane potential, action potential amplitude, force of contraction and rate of change in force.

B.2.6. Assessment of vascular smooth muscle function

The vascular reactivity of both the elastic/conduit and muscular/resistance arteries were assessed using established organ bath protocols [19, 21]. Thoracic aortas were cleaned and sectioned into five-millimetre-long segments. Tissues were anchored in 25 mL warmed (37ºC) organ bath containing gassed (O_2 95% / CO_2 5%) TPSS and connected to FT03 force displacement transducers (Grass Technologies, Middleton, WI, USA). The aortas were left to equilibrate for 30 minutes and set to a resting tension of 10 mN before being exposed to cumulative concentrations of acetylcholine, sodium nitroprusside and noradrenaline. The dosing cycle for each cumulative response curve (CRC) was as follows: 1×10^{-9} M, 3×10^{-9} M, 1×10^{-8} M, 3×10^{-8} M, 1×10^{-7} M, 3×10^{-7} M, 1×10^{-6} M, 3×10^{-6} M, 1×10^{-5} M, 3×10^{-5} M, 1×10^{-4} M and 3×10^{-4} M.

While submerged in TPSS, second-order mesenteric arteries were isolated, cleaned and sectioned into two-millimetre-long segments. Tissues were mounted into a multi-channel wire myograph system (DMT-Asia Pacific, Bella Vista, NSW, Australia) using 40-μm diameter stainless steel wire. Each chamber was slowly warmed to 37ºC following placement of the tissue and was constantly bubbled with carbogen gas (O_2 95% / CO_2 5%). Once temperature

had been reached, the mesenteric arteries were normalised to a transmural pressure of 100 mmHg [22]. Following a brief rest period, tissue viability was assessed by exposure to potassium chloride TPSS (all in mM: sodium chloride 37, potassium chloride 100, magnesium chloride 1, sodium dihydrogen phosphate 0.4, sodium bicarbonate 23, calcium chloride 2, glucose 6, ascorbic acid 0.3, ethylenediaminetetraacetic acid 0.05). Following this procedure, the mesenteric arteries were washed with TPSS and rested for 30 minutes before concentration-response curves to acetylcholine, sodium nitroprusside and noradrenaline were completed. The dosing cycle for each CRC was as follows: 1×10^{-9} M, 3×10^{-9} M, 1×10^{-8} M, 3×10^{-8} M, 1×10^{-7} M, 3×10^{-7} M, 1×10^{-6} M, 3×10^{-6} M, 1×10^{-5} M, 3×10^{-5} M, 1×10^{-4} M and 3×10^{-4} M.

In order to assess endothelial-dependent and -independent relaxation responses, the blood vessels were first constricted with a sub-maximal concentration of noradrenaline (1×10^{-6} M for thoracic aortas and 1×10^{-5} M for mesenteric arteries). Once a stable plateau had been reached (average magnitude of pre-contraction 3 mN for thoracic aortas and 7 mN for mesenteric arteries), the dosing cycle for acetylcholine, or sodium nitroprusside, was initiated. The results for the endothelium-dependent and -independent relaxation analyses were expressed as percentage relaxation of tone induced by noradrenaline. Non-linear regression was used to calculate $Log_{10}EC50$ values [19] and statistical analyses were performed as described in section B.3.2.

B.2.7. Assessment of gene expression

Reverse transcription PCR (RT-qPCR) was used to assess changes in mRNA levels of selected genes. Following euthanasia, tissues (including the left ventricle, kidneys, gastrocnemius, soleus and tibialis anterior) were isolated, snap-frozen in liquid nitrogen and stored at -80^{o}C

until the time of analysis. To extract the mRNA, 50 mg tissue samples were homogenised in Trizol™ reagent (Applied Biosystems Inc.). Following a brief incubation period, 100 µL of chloroform was added to each homogenate. The samples were subsequently centrifuged (15 minutes at 16 400 × *g* at 4ºC) and the aqueous phase was isolated. Following an overnight incubation in 250 µL of isopropanol at -20ºC, the samples were again centrifuged, and the supernatant was decanted. The resulting pellet was washed three times with ethanol and left to air-dry for at least 10 minutes. The samples were resuspended in 20-50 µL of RNase-free water and mRNA yield was assessed using spectrophotometry (Nanodrop™ 2000, Thermofisher Scientific).

Samples were reversed transcribed to cDNA using SuperScript™ III Reverse Transcriptase (RT), as per the manufacturer's instructions (Applied Biosystems Inc.). Non-template controls and no-RT controls were made by substituting template mRNA or RT with RNAse-free water, respectively. Once synthesised, a 2 µL aliquot of each cDNA sample was dispensed into 0.1 mL strip tubes (in duplicate), along with 5 µL of TaqMan® Fast Advanced Master Mix, 0.5 µL of TaqMan® Gene Expression Assay (details of the specific assays used in each study are provided in the corresponding chapters) and 4.5 µL of RNase-free water. Strip tubes were capped and loaded into a Rotor-Gene Q system (Qiagen) for the PCR reaction, as per the following conditions: 50ºC for 2 minutes; 95ºC for 20 seconds and; 40 x cycles at 95ºC for 3 seconds (denature) and 60ºC for 30 seconds (anneal/extend). Samples with *Ct* values > 35 were excluded and no-RT controls were acceptable if *Ct* values were $\geq$ five cycles compared to samples [23]. The average *Ct* value was calculated for each duplicate pair and relative expression was determined according to the delta-delta C*t* method [24].

B.2.8. Assessment of serum biomarkers

Blood samples were collected from the abdominal vena cava following euthanasia. The samples were left to clot before centrifugation at 3000 × *g* for 15 minutes. The resulting supernatant (i.e. serum) was removed and stored at -80^{o}C until the time of analysis. Selected biomarkers of skeletal muscle, cardiovascular and/or renal integrity were measured using a Roche Diagnostics Cobas Integra® 400 Plus Biochemical Analyser (the specific biomarkers assessed in each study are reported in the corresponding chapters). Samples were defrosted overnight in a refrigerator and aliquots (either undiluted or diluted in milli-Q water) were dispensed into clean cupules for analysis. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were also used for the assessment of skeletal muscle biomarkers (Fisher Biotec, Wembley, Australia). These assays were performed according to the manufacturer's protocols.

B.2.9. Assessment of skeletal muscle hydroxyproline content

Tissue hydroxyproline content was measured using a colourimetric assay adapted from Reddy and Enwemeka [25]. For each muscle, 0.2 g of tissue was isolated and homogenised in milli-Q water. A 38 µL aliquot of homogenate was placed into a centrifuge tube and spiked with 2 µL of 1 mg/mL hydroxyproline stock solution. This stock was also used to make a series of standards ranging in concentration from 0 – 50 mg/mL. All samples and standards were hydrolysed by being heated to 110^{o}C (for 25 minutes) following the addition of 10 µL of 2 M sodium hydroxide. The samples/standards were left to cool to room temperature before 450 µL of chloramine T-reagent was added to each hydrolysate. The samples/standards were left to stand for 25 minutes, then 100 µL aliquots were taken from each tube and dispensed into separate wells of a 96-well plate (in duplicate). Following the addition of 100 µL of freshly

prepared Ehrlich's aldehyde reagent to each well, the plate was incubated at 65ºC for 20 minutes. The absorbance of all samples/standards was then read at 540 nm using a plate reader (Multiskan EX, Thermo Scientific™).

B.3. Additional information

B.3.1. *A priori* power analyses

In order to determine the sample sizes to be used in this study, *a prior* power analyses were conducted using G*Power (Heinrich Heine University, Düsseldorf, Germany). Effect sizes from pilot study datasets were used for the calculations. These results of the *a priori* analyses were as follows:

- Isolated skeletal muscle organ baths: 18 animals per group (two-tailed t-test between independent means, alpha level 0.05, power beta level of 0.8, effect size 0.98).
- Single-cell microelectrode studies: 11 animals per group (f-test fixed effects one-way, alpha level 0.05, power beta level of 0.8, effect size 0.58).
- Reverse transcription PCR: 4 animals per group (two-tailed t-test between independent means, alpha level 0.05, power beta level of 0.8, effect size 2.5).

The calculations indicated that a minimum of 18 rats per group would be required. However, larger samples sizes were used during this project as both single-cell microelectrode studies and Langendorff heart preparations had to be performed. Specifically, as these experiments could not be completed on the same heart, a minimum of 22 animals per group was required (i.e. the sample size calculated for the single-cell microelectrode studies was doubled).

B.3.2. Statistical analysis

Details of the statistical testing conducted in each study are reported in the corresponding chapters, but a brief account of these analyses is provided here. Prior to statistical testing, all data was subjected to ROUT analysis for the identification of potential outliers. The Q value for the ROUT test was set to 5% (i.e. fewer than 5% of statistically significant datapoints to be false positives) [26]. If identified as an outlier, data for the affected variable only, rather than the whole rat, was excluded. Outliers were removed in this manner as the terminal experiments were performed *ex vivo* on isolated tissues. Thus, anomalous results may have been due to issues with tissue preparation/experimental factors, rather than an innate physiological difference in the rat. Data for inviable tissues (i.e. those that did not respond during experimental testing) were also removed prior to statistical analysis. Following outlier evaluation, data were checked for normality using histograms, skewedness and kurtosis statistics, as well as the D'Agostino-Pearson or Kolomogov-Smirnov test [27]. Normally distributed data was evaluated using one-way analysis of variance (ANOVA), or repeated measures two-way ANOVA, followed by Tukey post-hoc testing as appropriate. If data was not normally distributed, the Kruskal-Wallis test (with Dunn's post-hoc test), or the Scheirer-Ray-Hare test, was used. Significance was set at an alpha level of 0.05, and all statistical tests were performed using GraphPad Prism 7 or 8 (GraphPad Software, La Jolla, USA). Unless otherwise stated, groups of individual responses were presented as mean values with standard deviations (SD).

B.3.3. Reagents and chemicals

Unless otherwise stated, all reagents and chemicals were of analytical grade and sourced from Merck (Australia) or Thermofisher Scientific (Australia). Noradrenaline (99%,), acetylcholine ($\geq$ 99%) and sodium nitroprusside ($\geq$ 99%) were purchased from Merck (Australia).

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APPENDIX C

Tabular summary of skeletal muscle mRNA expression results

Preamble

Appendix C provides a tabular summary of the skeletal muscle mRNA expression data collected during this project. Although these results are reported and discussed elsewhere in this thesis, they are reproduced in this Appendix for ease of comparison.

D. mRNA expression results

D.1. Gastrocnemius

Table D.1.1. Gastrocnemius mRNA expression results for SIM80 rats

Gene of interest	Chapter 3[†]	Chapter 4	Chapter 5	Chapter 6
Atrogin-1	↑ vs. CON	—	—	—
Ctsl	N/A	—	N/A	N/A
MHC type I	N/A	—	N/A	N/A
MHC type IIA	↓ vs. CON	—	N/A	N/A
MHC type IIB	N/A	—	N/A	N/A
MHC type IIX/D	N/A	—	N/A	N/A
Mstn	N/A	—	—	N/A
Mt1a	N/A	—	—	—
Murf-1	N/A	—	N/A	—
Nox2	N/A	—	—	N/A
Pdk4	N/A	—	—	—
Pgc1α	N/A	—	—	N/A
Pparα	N/A	—	—	—
Sod1	N/A	N/A	—	N/A
Sod2	↑ vs. CON	—	N/A	—
Tnfα	N/A	N/A	—	N/A
Ucp3	N/A	—	N/A	N/A

[†] CON refers to CON80; — denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression; ↑ denotes a statistically significant increase in expression; N/A denotes that mRNA levels were not assessed in the study.

Table D.1.2. Gastrocnemius mRNA expression results for SIM40 rats

Gene of interest	Chapter 4
Atrogin-1	—
Ctsl	—
MHC type I	—
MHC type IIA	—
MHC type IIB	—
MHC type IIX/D	—
Mstn	—
Mt1a	—
Murf-1	—
Nox2	↓ vs. SIM80
Pdk4	—
Pgc-1α	—
Pparα	—
Sod2	—
Ucp3	—

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression.

Table D.1.3. Gastrocnemius mRNA expression results for PRAV160 rats

Gene of interest	Chapter 5
Atrogin-1	—
Mstn	—
Mt1a	↓ vs. SIM80
Nox2	—
Pdk4	—
Pgc-1α	—
Pparα	—
Sod1	—
Tnfα	—

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression.

Table D.1.4. Gastrocnemius mRNA expression results for SIM50 rats

Gene of interest	Chapter 3[†]
Atrogin-1	—
MHC type IIA	↑ vs. CON
Sod2	—

[†] CON refers to CON50; — denotes no a statistically significant change in gene expression; ↑ denotes a statistically significant increase in expression.

Table D.1.5. Gastrocnemius mRNA expression results for CON+GGOH rats

Gene of interest	Chapter 6
Atrogin-1	—
Mt1a	—
Murf-1	—
Pdk4	—
Pparα	—
Sod2	—

— denotes no statistically significant change in gene expression.

Table D.1.6. Gastrocnemius mRNA expression results for SIM+GGOH rats

Gene of interest	Chapter 6
Atrogin-1	—
Mt1a	—
Murf-1	—
Pdk4	—
Pparα	—
Sod2	—

— denotes no statistically significant change in gene expression.

D.2. Soleus

Table D.2.1. Soleus mRNA expression results for SIM80 rats

Gene of interest	Chapter 3	Chapter 4	Chapter 5	Chapter 6
Atrogin-1	—	—	—	—
Ctsl	N/A	—	N/A	N/A
MHC type I	N/A	—	N/A	N/A
MHC type IIA	—	—	N/A	N/A
MHC type IIB	N/A	↓ vs. CON	N/A	N/A
MHC type IIX/D	N/A	—	N/A	N/A
Mstn	N/A	—	—	N/A
Mt1a	N/A	—	—	—
Murf-1	N/A	—	N/A	—
Nox2	N/A	—	—	N/A
Pdk4	N/A	—	—	—
Pgc1α	N/A	—	—	N/A
Pparα	N/A	—	—	—
Sod1	N/A	N/A	—	N/A
Sod2	—	—	N/A	—
Tnfα	N/A	N/A	—	N/A
Ucp3	N/A	—	N/A	N/A

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression; ↑ denotes a statistically significant increase in expression; N/A denotes that mRNA levels were not assessed in the study.

Table D.2.2. Soleus mRNA expression results for SIM40 rats

Gene of interest	Chapter 4
Atrogin-1	—
Ctsl	—
MHC type I	—
MHC type IIA	—
MHC type IIB	—
MHC type IIX/D	—
Mstn	—
Mt1a	↑ vs. SIM80
Murf-1	—
Nox2	—
Pdk4	—
Pgc-1α	—
Pparα	—
Sod2	—
Ucp3	—

— denotes no statistically significant change in gene expression; ↑ denotes a statistically significant increase in expression.

Table D.2.3. Soleus mRNA expression results for PRAV160 rats

Gene of interest	Chapter 5
Atrogin-1	—
Mstn	—
Mt1a	—
Nox2	—
Pdk4	—
Pgc-1a	—
Ppara	↓ vs. SIM80
Sod1	↓ vs. SIM80
Tnfa	—

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression.

Table D.2.4. Soleus mRNA expression results for SIM50 rats

Gene of interest	Chapter 3
Atrogin-1	—
MHC type IIA	—
Sod2	—

— denotes no statistically significant change in gene expression.

Table D.2.5. Soleus mRNA expression results for CON+GGOH rats

Gene of interest	Chapter 6
Atrogin-1	—
Mt1a	—
Murf-1	—
Pdk4	—
Ppara	—
Sod2	—

— denotes no statistically significant change in gene expression.

Table D.2.6. Soleus mRNA expression results for SIM+GGOH rats

Gene of interest	Chapter 6
Atrogin-1	—
Mt1a	—
Murf-1	—
Pdk4	—
Ppara	—
Sod2	—

— denotes no statistically significant change in gene expression.

D.3. Tibialis anterior

Table D.3.1. Tibialis anterior mRNA expression results for SIM80 rats

Gene of interest	Chapter 3	Chapter 4	Chapter 5	Chapter 6
Atrogin-1	—	—	—	—
Ctsl	N/A	—	N/A	N/A
MHC type I	N/A	—	N/A	N/A
MHC type IIA	—	—	N/A	N/A
MHC type IIB	N/A	—	N/A	N/A
MHC type IIX/D	N/A	—	N/A	N/A
Mstn	N/A	—	—	N/A
Mt1a	N/A	—	—	—
Murf-1	N/A	—	N/A	—
Nox2	N/A	—	—	N/A
Pdk4	N/A	—	—	—
Pgc1α	N/A	—	—	N/A
Pparα	N/A	—	—	—
Sod1	N/A	N/A	—	N/A
Sod2	—	—	N/A	—
Tnfα	N/A	N/A	↑ vs. CON	N/A
Ucp3	N/A	—	N/A	N/A

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression; ↑ denotes a statistically significant increase in expression; N/A denotes that mRNA levels were not assessed in the study.

Table D.3.2. Tibialis anterior mRNA expression results for SIM40 rats

Gene of interest	Chapter 4
Atrogin-1	—
Ctsl	—
MHC type I	—
MHC type IIA	—
MHC type IIB	—
MHC type IIX/D	—
Mstn	—
Mt1a	—
Murf-1	—
Nox2	↓ vs. SIM80
Pdk4	↑ vs. CON
Pgc-1α	—
Pparα	—
Sod2	↓ vs. SIM80
Ucp3	—

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression; ↑ denotes a statistically significant increase in expression.

Table D.3.3. Tibialis anterior mRNA expression results for PRAV160 rats

Gene of interest	Chapter 5
Atrogin-1	—
Mstn	—
Mt1a	—
Nox2	—
Pdk4	—
Pgc-1a	↓ vs. SIM80
Ppara	—
Sod1	—
Tnfa	—

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression.

Table D.3.4. Tibialis anterior mRNA expression results for SIM50 rats

Gene of interest	Chapter 3
Atrogin-1	—
MHC type IIA	—
Sod2	—

— no statistically significant change in gene expression.

Table D.3.5. Tibialis anterior mRNA expression results for CON+GGOH rats

Gene of interest	Chapter 6
Atrogin-1	—
Mt1a	—
Murf-1	—
Pdk4	↓vs. SIM80
Ppara	—
Sod2	—

— denotes no statistically significant change in gene expression; ↓ denotes a statistically significant decrease in expression.

Table D.3.6. Tibialis anterior mRNA expression results for SIM+GGOH rats

Gene of interest	Chapter 6
Atrogin-1	—
Mt1a	—
Murf-1	—
Pdk4	—
Ppara	—
Sod2	—

— denotes no statistically significant change in gene expression.