Investigation of the Association between Genetics, Drug Exposure and Statin-induced Muscle Toxicity

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ABSTRACT

Statins are generally well-tolerated, although statin-related myotoxicity (SRM) has been reported in a considerable number of patients. The risk factors underlying SRM have yet to be fully characterised. This study aimed to further elucidate the risk factors that increase the likelihood of SRM using data generated from cellular and clinical settings. The data from the cellular studies would provide information regarding candidate single nucleotide polymorphisms (SNPs) which could be tested in the clinical setting.

In the cellular studies, three model cell lines were used; human proximal tubule (HK-2), rat skeletal muscle (L6) and human muscle cells. Lipophilic statins, simvastatin and atorvastatin, inhibited monocarboxylate transporter 1 (MCT1)-mediated DL-lactate uptake at the same magnitude as phloretin, a well-known MCT1 inhibitor. No significant lactate uptake inhibition was observed with up to 1 mM of hydrophilic statins (pravastatin and rosuvastatin). The magnitudes of inhibition of multidrug resistance-associated protein (MRP)-mediated CMFDA efflux and MDR1-mediated Hoeschst 33342 efflux by the lipophilic statins were lower than that caused by MK571 and cyclosporine A, which are typical inhibitors of MRP and MDR1, respectively. Both hydrophilic statins showed no significant effect on MRPs and MDR1 functions.

In the clinical setting, a case-control study (116 cases and 314 controls) of unrelated dyslipidaemic patients was performed to determine the association between 12 SNPs from nine focus genes [i.e., SLCO1B1, ABCC2, ABCG2, CYP3A4 (*22 allele), COQ2, GATM, GPx, SLC16A1, SLC16A3] and SRM. Of the 12 SNPs genotyped, only SNP in SLCO1B1 (rs4149056) appeared to be the most important genetic predictor of SRM ($P = 0.059$, $P = 0.047$ in univariate and multivariate analysis, respectively), thus confirming previous findings. The association between rs4149056 and SRM was demonstrated to be independent to the type of administered statins and was likely to be influenced by the patient gender. Further patient recruitment is ongoing to increase study power and to confirm the assumption of the abovementioned association.

Abbreviations: ABCC2, ATP-binding cassette, subfamily C member 2; ABCG2, ATP-binding cassette, subfamily G member 2; CMFDA, 5-chloromethylfluorescein-diacetate; COQ2, Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase; CYP3A4, Cytochrome P450 subfamily 3A, member 4; GATM, Glycine aminidotransferase; GPx, glutathione peroxidase; MDR1, Multidrug resistance protein 1; SLCO1B1, Solute carrier organic anion transporter family member 1B1.

PUBLICATIONS RESULTING FROM THE THESIS

1. Jenkinson, S. E., Chung, G. W., van Loon, E., Bakar, N. S., Dalzell, A. M. and Brown, C. D. A. (2012). The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule, *Pflügers Archiv - European Journal of Physiology*, 464(6), pp. 601-611.

2. Bakar, N. S, Jenkinson, S. E, Kamali, F. and Brown C. D. A. (2012). Statin Inhibition of Monocarboxylate Transporter (MCT)-Mediated Lactate Transport in HK-2 Cells. *Proceedings of the British Pharmacological Society* at http://www.pA2online.org/abstracts/Vol10Issue4abst085P.pdf

3. Nur Salwani Bakar, Dermot Neely, Peter Avery, Colin Brown, Ann Daly and Farhad Kamali. Evaluation of the effect of genetic, patient and clinical factors on statin-related myotoxicity: a case-control study (*manuscript in preparation*).

4. Nur Salwani Bakar, Farhad Kamali, Colin Brown. Effect of statins on functional expression of membrane transporters in L6 rat skeletal muscle cells (*manuscript in preparation*).

DECLARATION OF ORIGINALITY

The material contained in this thesis has not previously been submitted for a degree to Newcastle University, UK, or any other university. The research reported here has been conducted by the author unless indicated otherwise. All help given by others has been acknowledged and all sources of information are indicated in the text.

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ABBREVIATION LIST

µCi	Microcurie
µg	Microgram
µg/ml	Microgram per mililiter
µl	Microliter
µM	Micromolar
ABC	ATP-binding cassette
ABCC2	ATP-binding cassette, sub-family C member 2
ABCG2	ATP-binding cassette, sub-family G member 2
ACC/AHA/NHLBI	American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute
ACE	Angiotensin-converting enzymes
ADR	Adverse drug reaction
ALP	Alkaline phosphatase
Apo E	Apolipoprotein E protein
ATP	Adenosine triphosphate
AUC	Plasma drug concentration versus time area under the curve
BCRP	Breast cancer resistance protein
BMI	Body mass index
BSEP	Bile Salt Export Pump
$CaCl_2$.2H2O	Calcium Chloride Dihydrate
CETP	Cholesteryl ester transfer protein
CEU	Caucasians

CHC	α-Cyano-4-hydroxycinnamic acid *N*-ethyl-*N*,*N*-diisopropylammonium salt
CHD	Coronary heart diseases
CI	Confidence interval
CK	Creatine kinase
cm^2	Centimeter squared
CMFDA	5-chloromethylfluorescein-diacetate
CNS	central nervous system
COQ2	Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase
CoQ_{10}	Coenzyme Q_{10} (ubiquinone)
CSA	Cyclosporin A
CVD	Cardiovascular diseases
CYP	Cytochrome P450
DDI	drug-drug interaction
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DPM	Disintegrations per minute
Em	Emission
Ex	Excitation
FAM	Fluorescein amidite
FDA	Food and Drug Administration

FPP	Farnesyl pyrophosphate (FPP)
g	Gram
GATM	Glycine aminidotransferase
GGPP	Geranylgeranyl pyrophosphate
GPx	Glutathione peroxidase
GMSF	Glutathione methylfluorescein
GWAS	Genome-wide association study
H33342	Hoechst 33342
HDL-C	High-density lipoprotein cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Het	Heterozygous
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HK-2	Human proximal tubule cells
HLA	Human leukocyte antigen
L6	Rat skeletal muscle cells
LD	Linkage disequilibrium
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDL-C	LDL-cholesterol
KCl	Potassium chloride
kDa	Kilodalton

KH_2PO_4	Potassium dihydrogen phosphate
K_m	Michaelis-Menten kinetic
MAF	Minimum allele frequency
MCT	Monocarboxylate transporter
MDR1	Multidrug resistance protein 1 (P-glycoprotein)
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke
MEM	Minimum essential medium
$Mg.SO_4.7H_2O$	Magnesium sulfate heptahydrate
MK571	5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid
ml	Mililiter
mM	Milimolar
MnSOD	Manganese superoxide dismutase
MRP2	Multidrug resistance-associated protein 2
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTTP	Microsomal triglyceride transfer protein
Mut	Mutant
NaCl	Sodium chloride
ng/ml	Nanogram per mililiter
$NH_2PO_4.2H_2O$	Sodium phosphate monobasic dihydrate
nm	Nanometer
NTCP	Na^+-taurocholate cotransporting polypeptide

OATP	Organic anion-transporting polypeptide
OR	Odds ratio
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PD	Pharmacodynamics
Pen/strep	Penicillin/Streptomycin
P-gp	P-glycoprotein
PK	Pharmacokinetics
PRIMO	Prediction of Muscular Risk in Observational Conditions
RCT	Randomised controlled trial
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rs	Ref Seq
SAGE	Study Assessing Goals in the Elderly
SDS	Sodium dodecyl sulphate
SLC16A	Solute carrier family 16A
SLCO1B1	Solute carrier organic anion transporter family member 1B1
SNP	Single nucleotide polymorphism
SRM	Statin-related myotoxicity
TAE	Tris/ Acetic acid /EDTA buffer

TEMED	Tetramethylethylenediamine
ULN	upper limit of normal
VLDL	Very low density lipoprotein
WT	Wild type

Chapter 1. Introduction

1.1 Atheroslerosis and Lipid Lowering Drugs

Hypercholesterolaemia is the major risk factor for atherosclerosis-related and cardiovascular disease (CVD) across the globe. A report released by the World Health Organization (WHO) that the leading cause of death in 2004 worldwide for both male and females was attributed to CVD with per cents of total deaths of 31.5% and 26.8%, respectively (World Health Organisation, 2008). Familial hypercholesterolaemia (FH) is a common genetic cause of premature coronary heart disease (CHD) (i.e. ischaemic heart disease), such as myocardial infarction and angina pectoris. This is due to lifelong elevated plasma low-density lipoprotein (LDL) cholesterol levels (Austin et al., 2004, Goldstein et al., 2001). In the UK, CHD are the main cause of death and accounted for almost 180,000 deaths in 2010 ~ approximately one in three of all deaths that year (Townsend et al., 2012). However, the consequence of missed diagnosis and under-treatment of individuals in the general population with FH, which attributed to the CHD, is largely unknown (Nordestgaard et al., 2013). Although the reliable number of individuals diagnosed with FH is difficult to predict, it was estimated that the condition accounted for nearly 12 % (approximately 123, 600 individuals) of overall UK population (Nordestgaard et al., 2013).

Atherosclerosis is the most prevalent disease responsible for death from myocardial infarction, cerebrovascular events and renal failure. Atherosclerosis derives from the Greek language: *athere* meaning soft paste-like material and *sclero* for hard, a result from the proliferation of fibrous tissue and the presence of calcification (Fernandes e Fernandes et al., 2014). Its pathogenesis is complex and multifactorial and several epidemiological studies have identified risk factors, which are associated with its severity and widespread involvement of the arterial tree. Smoking, hypertension, hypercholesterolaemia, and diabetes mellitus have all been associated with atherosclerosis and the mechanisms leading to arterial injury became clear in extensive research conducted in the last two decades. The association between fatty materials such as cholesterol and atherosclerosis has been proven epidemiologically in human studies (De Backer et al., 2003, Cleeman et al., 2001). Injury and subsequently narrowing of the arteries lumen result from the deposition of macrophages laden with lipids, mainly oxidised LDL and formation of the atherosclerotic plaque. This atheromatous plaque can act as a focus for localised thrombosis or as a source of distal embolisation. By causing arterial stenosis or

thrombosis, atherosclerosis can eventually lead to regional ischemic damage, which can be life threatening.

Treatment for atherosclerosis varies depending on the clinical manifestation of the disease. Some patients may opt for maintaining low fat diet and regular exercise, and some may require surgical intervention such as endarterectomy (a surgical procedure to remove the atheromatous plaque material or blockage) or drugs treatment. Lipid-modifying interventions have been shown to decrease the risk of coronary heart disease both in patients with hypercholeterolaemia and those with relatively normal level of LDL-cholesterol (LDL-C). Expert panels in the USA and Europe have also recommended dietary changes and, if necessary, the lipid-modifying therapy to decrease elevated cholesterol concentrations, particularly LDL-C (De Backer et al., 2003, Cleeman et al., 2001).

Lipid lowering drugs, such as statins, are the first-line option and have been demonstrated to significantly lower the incidence of CVD in at risk individuals (Corsini, 2003, Sever et al., 2003), whilst other lipid-modifying drugs such as bile acid-binding resins (e.g. cholestyramine, colestipol, colesevalam), nicotinic acid (niacin), fibric acid derivatives (e.g. fenofibrate, clofibrate, gemfibrozil, bezafibrate), and more recently the cholesterol-absorption inhibitors (e.g. ezetimibe) are also used to treat hypercholesterolaemia. The effect of statins on lowering the progression of coronary atherosclerosis, resulting in fewer new lesions and total occlusions compared with untreated hypercholesterolaemic patients, has been independently demonstrated by several investigators (Smilde et al., 2001, Vaughan et al., 2000, Christians et al., 1998). This has been suggested to be a consequence of the shrinkage of the lipid core of the atherosclerotic plaque, avoiding plaque rupture that would otherwise trigger intramural haemorrhage and intraluminal thrombosis (Christians et al., 1998).

The target organ for statins action is liver, the vital organ in human body that has wide range of functions including lipid metabolism and cholesterol synthesis. In the liver, the mechanism of statins in lowering LDL cholesterol plasma concentrations starts with the inhibition of HMG-CoA reductase thus inhibiting HMG-CoA conversion to mevalonate. The latter is the rate-limiting step in *de novo* cholesterol biosynthesis in the liver (**Figure 1.1**). The consequent drop in cholesterol synthesis in hepatocytes leads to increased expression of LDL receptors (as a compensatory mechanism) in order to maintain

adequate intra-hepatic cholesterol levels (Lennernas and Fager, 1997, Brown and Goldstein, 1986) The increased expression of LDL receptors on hepatocytes will subsequently lead to the increased uptake of LDL from plasma. This also results in increased extraction of LDL-C from the blood and subsequently decreased circulating total cholesterol and LDL-C concentrations. As a result, statins prevent incidence and recurrence of cardiovascular disease because of reduction of plasma LDL.

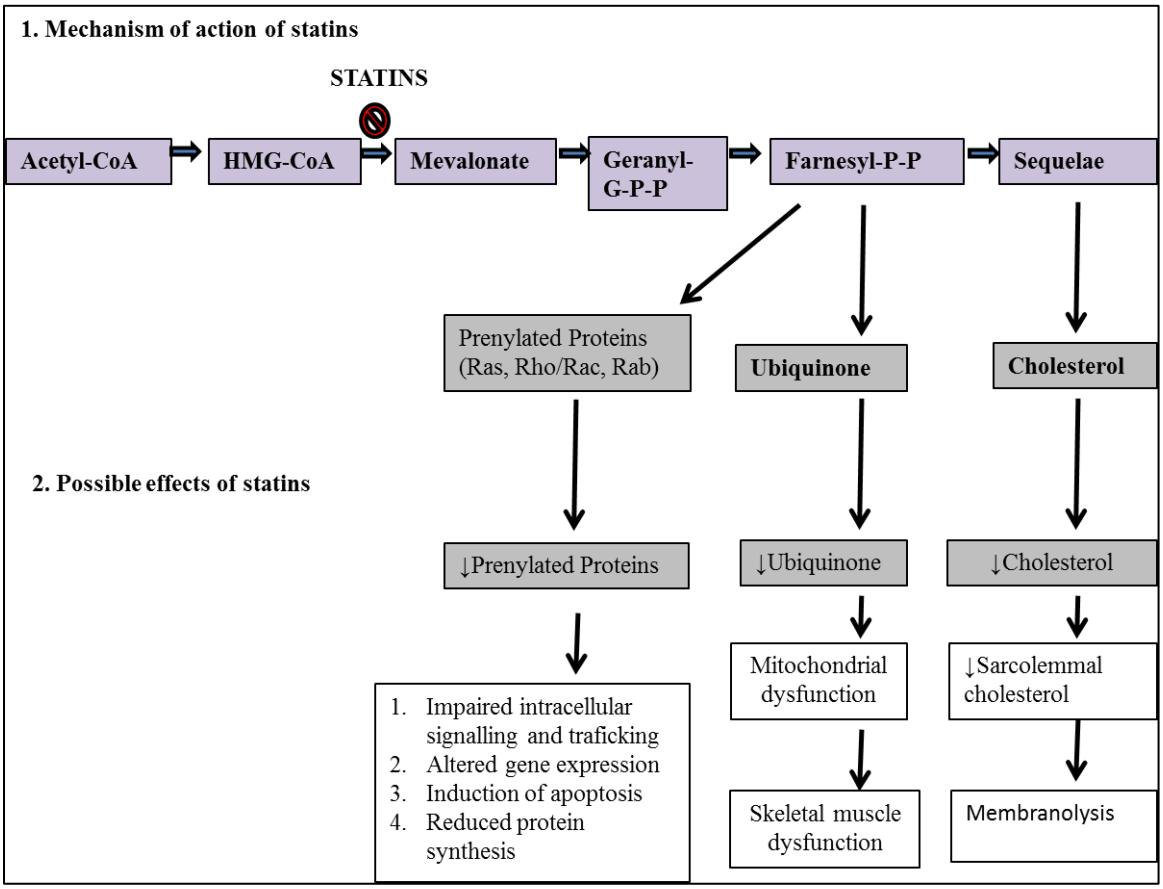


Figure 1.1 Biosynthesis pathways of cholesterol and downstream effects by statins (adapted from Needham & Mastaglia, 2014)

1.2 Statins

1.2.1 Clinical benefits of statins

Over the last decade, there has been a considerable number of large-scale clinical trials carried out to demonstrate the beneficial effects of statins in reducing morbidity and mortality in patients with and without CVD (Downs et al., 1998, Scandinavian Simvastatin Survival Study Group, 1994, Shepherd et al., 1995). Statins have been proven to improve survival rate for patients who were at risk but without established cardiovascular disease (Brugts et al., 2009). They were also shown to reduce stroke events by 10% with 1 mmol/l decrease in LDL-C concentration and up to 17% with a 1.8 mmol/l reduction (Law et al., 2003). According to a systematic review using various evidence-based clinical reports, the highest doses of approved statins have been shown to decrease LDL-C- concentration in blood; 63 % with 40 mg rosuvastatin, 57 % with 80 mg atorvastatin, 46 % with 80 mg simvastatin, 41 % with 4 mg pitavastatin, 40 % with 80 mg lovastatin, 34 % with 80 mg pravastatin, and 31 % with 80 mg fluvastatin (Smith et al., 2009). Statins also affect other lipid parameters, including increasing high-density lipoprotein cholesterol (HDL-C) concentration and decreasing triglyceride concentration (Maron et al., 2000). Secondary mechanisms initiated by statins which reduce levels of atherogenic lipoproteins have also been reported. For instance, inhibition of hepatic synthesis of apolipoprotein (Apo) B100 and a reduction in the synthesis and secretion of triglyceride-rich lipoprotein i.e., VLDL and IDL, was shown with statins uptake (Grundy, 1998, Ginsberg et al., 1987).

Several studies have also revealed that the effects of statins were not just due to their lipid lowering capacity; statins also possess some pleiotropic effects, independent of their lipid modifying properties (Liao, 2002) including restoration of endothelial cell function, modification of inflammatory responses, antithrombotic and antioxidant effects, as well as reduction of smooth muscle cell proliferation and cholesterol accumulation (Fang et al., 2013, Bonetti et al., 2002, Farmer, 2000) all of which contribute to the clinical benefits of statins. Indeed, in recent years a substantial quantity of data has accumulated showing that statins exert various effects on multiple targets, which are independent of their plasma cholesterol lowering properties. Many of these clinical effects (or so-called pleiotropic effects) have been shown to be secondary to the inhibition of the synthesis of isoprenoid intermediates of the mevalonate pathway (Goldstein and Brown, 1990), such

as farnesylpyrophosphate (Farnesyl-P-P) and geranylgeranylpyrophosphate (Geranyl-G-P-P) and, therefore, are completely independent of the intracellular cholesterol biosynthesis (**Figure 1.1**). The inhibition of Farnesyl-P-P and Geranyl-G-P-P biosynthesis, which then leads to a reduction in the prenylation of small GTP-binding proteins such as Ras, Rac and Rho which are involved in muscle cell apoptosis (see **figure 1.1**). Furthermore, Farnesyl-P-P is an intermediate metabolite of ubiquinone/ coenzyme Q10 (CoQ10), a steroid isoprenoid, which plays an important role in the cellular energy transduction in the mitochondrial electron transport system. Thus, a reduced CoQ10 level may be one of the reasons underlying statin-related muscle adverse effects, consistent with the evidence of myofiber atrophy and muscular dystrophy shown as a result of a reduction of serum CoQ10 level (Miles et al., 2005). These biological effects beyond LDL reduction may differ among statins (Bonetti et al., 2003).

Independent to the cholesterol biosynthesis pathway, statins have also been shown to induce antiatherosclerotic effects by a series of mechanisms involved in their effect on lipids, which then lead to affect arterial myocytes, macrophages and metaloproteases (Shitara and Sugiyama, 2006), the inflammatory mediators that have a central role in atherosclerotic lesion development and thrombogenicity. Macrophage activation results in the excretion of proinflammatory and cytotoxic substances (Nathan, 1987), including peroxynitrite, an early inducer of atherosclerosis through the endoplasmic reticulum (ER) stress pathway (Dickhout et al., 2005). Further, the accumulation of free cholesterol or uptake of oxidized LDL induces macrophage apoptosis (Li et al., 2006). Cytokine release from macrophages augments the inflammatory response and increases the lesion size. Cytotoxic substances, including peroxynitrite and tumor necrosis factor (TNF)-α, released by the macrophage results in cell death of lesion-resident endothelial and smooth muscle cells, thereby disrupting vessel structure. These aspects of the macrophage in atherosclerotic lesion biology have been hypothesised to drive the progression of other chronic progressive diseases characterised by cell apoptosis and tissue fibrosis such as cytokine-driven disease, Type 2 Diabetes Mellitus, Alzheimer's disease and heart failure during statin therapy (Forrester and Libby, 2007). The possible association of diabetes, particularly, with statin therapy has started a wave of discussion in the medical community. A number of meta-analyses conducted in recent years have demonstrated that the association is real although causality has not been proved yet (Navarese et al., 2013, Preiss et al., 2011, Sattar et al., 2010, Rajpathak et al., 2009, Coleman et al., 2008).

1.2.2 Pharmacological properties of statins

Depending on their chemical structures, statins have different affinities for HMG-CoA reductase, which determine their pharmacological effects, and different pharmacokinetics (PK) properties. These include tissue distribution, metabolic stability, enzymes and the transporters involved in their metabolism (Shitara and Sugiyama, 2006). Some PK properties of the individual statins are summarised in **table 1.1**. Lipophilic statins are statins with high affinity of statins for a lipophilic environment and most of them show hepatoselective properties. Of all the lipophilic statins; cerivastatin has the highest octanol-water coefficient, an indication of the greatest degree of lipophilicity (McTaggart et al., 2001, Corsini et al., 1999). It therefore achieves higher levels of exposure in non-hepatic tissues and abundantly distributed over the peripheral tissue by passive diffusion (Hamelin and Turgeon, 1998). Furthermore, the lipophilic statins appear to be more susceptible to oxidative metabolism by cytochrome P450 (CYP) enzymes than hydrophilic drugs (Schachter, 2005). Rosuvastatin and pravastatin, in contrast, are considered as hydrophilic because of the presence of methane sulphonamide group and polar hydroxyl in their chemical structure, respectively (McTaggart et al., 2001, McTavish and Sorkin, 1991). Both of them are lack of the affinity to CYP enzymes and target membrane transporters (Kitamura et al., 2008) to exert their cholesterol lowering effects.

Table 1.1 Physicochemical and pharmacokinetic properties of statins

Characteristics	Lovastatin	Simvastatin	Pravastatin	Fluvastatin	Atorvastatin	Rosuvastatin	Pitavastatin*
Daily dosage (mg)	20-80	10-80	20-80	40-80	10-80	10-40	1-4
Origin	Fungi	Semisynthetic	Fungi	Synthetic	Synthetic	Synthetic	Synthetic
Pro-drug	Yes	Yes	No	No	No	No	No
Solubility	Lipophilic	Lipophilic	Hydrophilic	Intermediate	Lipophilic	Hydrophilic	Lipophilic
CNS permeation	Yes	Yes	No	No	No	No	No
First-pass metabolism	CYP3A4	CYP3A4	Multiple way	CYP2C9	CYP3A4	Limited CYP2C9	Minimally CYP2C9
Protein binding (%)	95	95	50	98	90	90	96
Half-life (hours)	2-3	2-3	1-2	0.5-2	13-16	19	11
Hepatic elimination (%)	69	79	46	>68	Not available	63	Not available
Renal excretion (%)	30	13	60	<6	<2	10	2

CYP, cytochrome P450 enzyme; CNS, central nervous system (table adapted from Chatzizisis et al. 2010).

*Evidences obtained from Alagona, 2010

1.2.3 Adverse effects of statins

Although statins have been proven to be safe (Armitage, 2007), they possess a number of side effects. Meta analyses from 90 studies identified that muscular adverse effects were still ranked first among other outcomes following chronic therapy with statins, with odds ratio (OR) of 2.63 (95% CI 1.50 to 4.61) followed by risk of liver enzymes elevation and type 2 diabetes with OR 1.54 (95% CI 1.47 to 1.62) and 1.31 (95% CI 0.99 to 1.73), respectively (Macedo et al., 2014a). Indeed, the risk of muscular events related to the statin use has received greatest attention since the sudden case of cerivastatin withdrawal, due to its reportedly rhabdomyolysis-related death in August 2001 (Thompson et al., 2003). The absence of a common agreement in the definition of statin-induced muscle events has hampered the precise estimation of their true incidence. Because patients considered to be susceptible to statin-induced muscle toxicity are excluded from controlled clinical trials, the reported adverse event rates may underestimate the true rate of these adverse effects in an unselected patient population.

There are also other less common adverse effects of statins, such as cognitive loss, neuropathy, pancreatic and hepatic dysfunction, and sexual dysfunction, which have been reported in large epidemiologic studies and case reports (Backes and Howard, 2003, Sreenarasimhaiah et al., 2002, Pia Iglesias et al., 2001, Muldoon et al., 2000). However, epidemiologic studies, for example randomised controlled trials (RCTs), could misinterpret and underscore a critically important point relevant to rare drug adverse effects (ADRs) in general, which merits emphasis and has relevance to other reported statins adverse effects. In the case with statins for example, the true frequency of adverse effects of statins is difficult to quantify. The RCTs often underestimate the frequency of this ADR because patients with symptoms of intolerance are typically excluded during the run-in period (Abd et al., 2011, Psaty et al., 2008). A significant increase in rates of a problem on statin vs placebo in RCTs supports a causal link between that drug and the abovementioned adverse effects in some people. However, absence of an average significant increase, or even presence of a significant average reduction in the problem, does not preclude causal occurrence of that problem in some individuals (Golomb and Evans, 2008). Therefore, observational studies which could be either retrospectively or prospectively in the study design, would have been better represented the true clinical situation in the case with statin adverse effects in particular, and any drugs in general. A good example of this could be obtained from the case of cerivastatin that has had resulted

in its withdrawal in 2001. Underscoring the limitations of RCTs for identification of statin adverse effects, cerivastatin was withdrawn from the market due to excess risk of rhabdomyolysis, although no cases of rhabdomyolysis occurred among cerivastatin users in a meta-analysis of RCTs (McClure et al., 2007). In contrast, observational studies of real-world use reported that rhabdomyolysis occurred with substantially higher frequency on cerivastatin than other statins (Cziraky et al., 2006, Graham et al., 2004), particularly for cerivastatin in combination with fibrates (Graham et al., 2004). Above of these reported adverse effects related to statin therapy, absolute excess risk of these observed unintended adverse effects, however, was reportedly small compared to the beneficial effects of statins on major cardiovascular events following their long-term use (Lv et al., 2014).

1.3 Statin-Related Myotoxicity (SRM)

1.3.1 Definition

Research in SRM has been hampered to some extent by the lack of standardised nomenclature and phenotypic definitions. Previously in 2002, the American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute (ACC/AHA/NHLBI) came up with terminology used to classify statin-related muscle adverse effects, including asymptomatic increases in creatine kinase (CK), myalgia, myositis and rhabdomyolysis, as summarised in **table 1.2** below. An international expert workshop on statin-induced myotoxicity was convened in December 2013, to agree on more robust definitions and a minimum set of criteria to help with the identification of cases (Alfirevic et al., 2014). Adapted from the definitions recommended by the ACC/AHA/NHLBI, a numeric classification was then developed by the expert group to standardize the degree of severity of SRM based on a defined algorithm (SRM0 to SRM6), which described the clinical manifestation with and without CK elevation and/or evidence of muscle biopsy findings (**Table 1.3**).

As described in the recently proposed definitions of SRM, myotoxicity covers the whole spectrum of drug induced muscle adverse effects. Myalgia, specifically, is a patient reported symptom, whereas myopathy is reserved for those cases with evidence of muscle damage, usually with CK elevation although the xULN cut-offs vary, >4x ULN for

SRM3 and >10xULN for SRM4 (severe myopathy). The term myositis has been used in the past to denote muscle symptoms with CK elevations, but the consensus group agreed that this should be used only where there is histopathology evidence of an inflammatory infiltrate. The term myopathy was used in SEARCH study (Link et al., 2008), but this excludes SRM2, (the most common presentation in clinical practice) in which CK elevation was <4xULN.

Table 1.2 Different terms of skeletal muscle-related problems described by ACC/AHA/NHLBI (adapted from Pasternak et al., 2002).

Condition	Definition
Myopathy	General term to describe all skeletal muscle related adverse effects
Asymptomatic	CK elevation without muscle symptoms
Myalgia	Muscle pain or weakness without CK elevation
Myositis	Muscle symptoms with CK elevation typically <10xULN
Rhabdomyolysis	Muscle symptoms with CK elevation typically >10xULN, and with creatinine elevation (usually with brown urine and urinary myoglobin)

CK= creatine kinase; ULN= upper limit of normal

Table 1.3 Recent proposed numeric and phenotype classification for statin-related myotoxicity (adapted from Alfirevic et al., 2014).

SRM Classification	Phenotype	Incidence	Definition
SRM 0	CK elevation <4XULN	1.5-26%	No muscle symptoms
SRM 1	Myalgia, tolerable	0.3 to 33%	Muscle symptoms without CK elevation
SRM 2	Myalgia, intolerable	0.2-2/1,000 patients years	Muscle symptoms, CK <4xULN, complete resolution on dechallenge
SRM 3	Myopathy	5/100,000	Muscle symptoms, CK >4xULN <10xULN, complete resolution on dechallenge
SRM 4	Severe myopathy	0.11%	Muscle symptoms, CK >10 x ULN <50x ULN, complete resolution on dechallenge
SRM 5	Rhabdomyolysis	0.1-8.4/100,000 patients years	Muscle symptoms, CK >10xULN or >50x ULN with evidence of renal impairment
SRM 6	Autoimmune-mediated necrotising myositis	~2/million per year	HMGCR antibodies and expression in muscle biopsy, incomplete resolution on dechallenge

1.3.2 Mechanism of SRM

The exact mechanism of statin myopathy remains unknown. The consequent intracellular depletion of intermediate metabolites and end products from the mevalonate pathway following statin therapy are thought by some to be the main events in the cellular apoptosis resulting in statin-induced myotoxicity. Since there is no solid, unifying pathway to explain mechanisms involved in SRM, I will outline, as described by Mosshammer and colleagues recently (Mosshammer et al., 2014), the literature background involved in the pathogenesis of statin side-effects as follows.

Pharmacokinetic (PK) factors

Contributory components involved in determining statin PKs are (i) statin hepatic biotransformation to metabolite, (ii) uptake into hepatocytes and (iii) efflux transport into bile canaliculi (Taha et al., 2014). PK factors from hepatocytes become the central focus for mechanism studies underlying statin-induced myopathy as they are among principal determinants of statin plasma concentration. With regards to hepatic biotransformation by CYP metabolising enzymes, PK studies consistently show higher systemic exposure of statins with substrate affinity to CYP3A4 when coadministered with an inhibitor of the CYP3A4 (Jacobson, 2004, Fichtenbaum et al., 2002, Azie et al., 1998). More commonly, statin-associated myopathy and rhabdomyolysis have been frequently reported during concomitant treatment with an inhibitor of CYP3A4 (Rowan et al., 2009, Maxa et al., 2002, Omar and Wilson, 2002). Similarly, genetic polymorphism in the CYP3A4 enzymes has also resulted in PK changes of statins, and thereby SRM. This will be discussed in **section 1.5**.

With respect to membrane drug transporters, inhibition or single nucleotide polymorphism (SNP) in uptake transporters OATPIBI at the sinusoidal membrane, was shown to affect simvastatin PKs and to a lesser degree other statins (Niemi, 2010). Although, other uptake transporters such as OATP1B3, OATP2B1 and the Na^+-taurocholate cotransporting polypeptide (NTCP) (Nies et al., 2013, Niemi, 2010, Ho et al., 2006), are also involved in hepatic statin transport, simvastatin are not affected by them as much as OATP1B1 (Sirtori et al., 2012). Until now, polymorphism in SLCO1B1, the gene that encodes the OATP1B1, is an important PK predictor for almost all statins as they share this transporter (Pasanen et al., 2007, Konig et al., 2006, Pasanen et al., 2006).

The SLCO1B1 polymorphism has a large effect on the PKs of hydrophilic rosuvastatin, as it is not metabolised by the CYP450 system (Pasanen et al., 2007).

To a lesser extent, there is evidence also that the ATP- binding cassette (ABC) efflux transporters affect statin PKs as well. Among the efflux transporters in the liver, only P-glycoprotein (P-gp, ABCB1), multidrug resistance-associated protein 2 (MRP2, ABCC2) and breast cancer resistance protein (BCRP, ABCG2) (Nies et al., 2013, Niemi, 2010) are shown to be implicated. Apart from expression in the liver, these efflux transporters are involved in intestinal secretion of statins (Generaux et al., 2011, Niemi, 2010, Itagaki et al., 2008). The contribution of membrane drug transporters in the intestine in determining statins PKs however remains inconclusive and needs further investigation (Mosshammer et al., 2014).

Transporters in muscle cell membrane

Transport of statins into and out of the muscle cells involves uptake and efflux transporters expressed on the membrane. They are therefore assumed to modulate local statin exposure in skeletal muscle and influence the development of SRM. So far, uptake transporter OATP2B1 (SLCO2B1) and efflux transporters multidrug resistance-associated proteins 1 (MRP1, ABCC1), 4 (MRP4, ABCC4) and 5 (MRP5, ABCC5) have been shown to facilitate statin transport across the sarcolemmal membrane of human skeletal muscle fibers (Knauer et al., 2010). Although OATP2B1 is the only SLCO-group uptake transporter thus far found at the myocyte membrane level, the contribution of other uptake transporters such as monocarboxylate transporters (MCTs) from SLC group is unclear.

MCT1 and MCT3/MCT4 are present in rat and human muscle cells (Bonen et al., 1997). MCT1 is expressed predominantly in slow-twitch oxidative (type I) fibers and in small quantity in fast-twitch glycolytic (type II) fibers (Thomas et al., 2012, Pilegaard et al., 1999). Interestingly, MCT1 has been identified, along with lactate dehydrogenase (LDH), in cardiac and skeletal muscle mitochondria, in which they aid mitochondrial lactate oxidation and facilitate the "intracellular lactate shuttle" (Brooks et al., 1999). It is suggested that reduced function of MCT1 due to a genetic polymorphism may explain the mitochondrial dysfunction among statin myopathic patients as type I fibers (high MCT1 contents) typically display a two- to threefold higher mitochondrial density (Picard et al., 2012). This would in turn explain higher type I fiber-targeted muscular injury in rats with

cerivastatin treatment (Obayashi et al., 2011), which is consistent with the high MCT1 level in the fibers. This is in agreement with MCT1's involvement in lactate transport in skeletal muscle and mitochondria (Brooks, 2009, Hashimoto et al., 2006, Brooks et al., 1999, Bonen et al., 1997). The contribution of the transporter can thus be proposed as a predictor for statin-related myotoxicity and/or mitochondrial toxicity. However, statins have been only found targeting MCT4 in a number of *in vitro* studies (Kobayashi et al., 2006, Sirvent et al., 2005, Nagasawa et al., 2003), but there is not much evidence regarding the involvement of MCT1.

Intracellular effect

The mechanism of SRM at the intracellular level can be associated with mitochondrial disorder due to CoQ_{10} deficiency. One of the end products from cholesterol biosynthetic pathway is CoQ_{10}, the component required for oxidative phosphorylation and adenosine triphosphate production in the mitochondria to maintain cell integrity. A decrease in CoQ_{10} biosynthesis and thus energy depletion mediated by statins has been postulated to be responsible for statin myotoxicity. To this effect, statin-mediated reduction of circulating, but not intramuscular CoQ_{10} concentrations, has been reported in both man and rats (Folkers et al., 1990, Willis et al., 1990). Statin therapy may reduce CoQ10 production, however, it is not clear whether or not statins reduce muscle CoQ10 concentrations (Marcoff and Thompson, 2007). Therefore it is uncertain whether CoQ_{10} reduction is involved in the pathophysiology of SRM. To a certain extent, CoQ_{10} deficiency may represent a predisposing factor for statin mediated myopathy, possibly in combination with other CoQ10 depleting conditions such as hereditary mitochondrial (e.g., MELAS syndrome) and metabolic disorders (Vladutiu et al., 2006). This proposal is supported by at least three cases of MELAS, syndrome characterised by mitochoncdrial myopathy, encephalopathy, lactic acidosis and stroke like episodes, after initiation of statin therapy in previously asymptomatic patients (Tay et al., 2008, Thomas et al., 2007, Chariot et al., 1993). Furthermore, findings from muscle biopsy suggest that statins could induce a mitochondrial myopathy. Indeed, ragged red fibers which are a characteristic of mitochondrial myopathy, have been reported in symptomatic patients on statin therapy (Phillips et al., 2002).

1.4 Non-genetic Risk Factors of SRM

Recently, Taha and colleagues (Taha et al., 2014) have classified broadly the factors associated with SRM into environmental and genetic risk factors. In this section, statin properties and its interactions with other concurrent medications, both of which considered as environmental factors, will be described.

1.4.1 Patient characteristics

Although the prevalence of statin-induced myopathy is considered low with only 1.2 per 10,000 persons/years, the risk may be increased by factors such as the patients' characteristics, concurrent drug medications and statin properties as discussed previously (Chatzizisis et al., 2010). In term of patient characteristics, several epidemiological studies have demonstrated that certain demographic features including advanced age (particularly above 80 years), female sex, small body frame and frailty, increase the risk of statin muscular adverse effects (Armitage, 2007, Schech et al., 2007, Pasternak et al., 2002). The incidence of drug adverse effects is increasing with age (> 65 years old) has been reported in a number of studies such as in the Study Assessing Goals in the Elderly (SAGE study) (Deedwania et al., 2007) and the MRC/BHF Heart Protection Study (Heart Protection Study Collaborative Group, 2002). Nevertheless, myopathic symptoms may be hard to discern from muscular complaints commonly experienced in elderly patients, making the interpretation of the exact proposed age for the predisposing risk of SRM difficult. Multiple drug therapy and age-related impairment of kidney function, to some extent, may exacerbate the risk of myopathy among elderly patients.

Females are more susceptible to SRM, by approximately 2-fold, because of their smaller vascular volumes and lower muscle mass, resulting in greater statin tissue exposure (Feng et al., 2012). Indeed, the risk of rhabdomyolisis was found more than 2-fold higher in females (Schech et al., 2007). On the other hand, a meta-analysis of 18 RCTs of statins with gender-specific outcomes did not detect any gender-specific differences in statin-related adverse events, probably because of the underrepresentation of women in the clinical trials concerned. Furthermore, only statin dosage, but not patient's gender, was identified as a risk factor for SRM in the Prediction of Muscular Risk in Observational Conditions (PRIMO) study (Bruckert et al., 2005). However, a risk-based approach according to gender factor has been used as selection criteria for cholesterol lowering

effect in Framingham study in which different age range for men and women; i.e.35-74 years old and 45-75 years old, respectively for safety concern (Anderson et al., 1991). This suggests gender, to a certain extent, also attributes to the risk of CHD diseases as well as the risk for statin adverse effects.

Apart from the age and gender of an individual, ethnic difference has also been regarded as a risk factor for SRM. The term ethnicity is a multidimensional classification that encompasses shared origins, social background, culture, and environment (Senior and Bhopal, 1994). It is generally recognised that inter-individual variation in statin response among Asians is likely attributed to the genetic make-up in drug metabolising enzymes and drug transporters (Chatzizisis et al., 2010). Asian subjects which include Chinese, Malaysian, and Indian had greater plasma drug concentration versus time area under the curve (AUC) of rosuvastatin in comparison to patients of European ancestry receiving similar doses (Lee et al., 2005). It is suggested that smaller body size in Asians may explain the underlying variability in drug response in some studies (Xie et al., 2001). Considering the ethic difference, the FDA has limited the administration of statin doses based on major continental race (Feng et al., 2012) with the recommendation that Japanese or Chinese descent administered a lower statin dosage i.e., below the one-half of the maximum dosage approved for use in the United States (Talbert, 2006).

1.4.2 Dosage and properties of statins

The dosage and physicochemical properties of statins are other risk factors of statin myopathy. As with other drug therapy, the risk for muscular adverse event is generally thought to be affected with higher statins dose (Davidson et al., 1997). In other word, patients who are exposed to high-dose statin therapy may be at higher risk. However, no linear relationship between plasma statin concentrations and the risk of adverse muscular events has been established. The overall risk of myopathy attributed to standard statin doses is reported to be typically low (<0.01%) (Armitage, 2007). A recent trial evaluating treatment with a standard dose of rosuvastatatin (20mg daily) reported that the rate of myopathy following statin treatment was no different to that of the matching placebo (Ridker et al., 2008). In case of higher statin dose regimens, increased risk of myopathy has been shown with high doses of simvastatin (160 mg daily) (Davidson et al., 1997) and pravastatin (160 mg daily) (Rosenson and Bays, 2003). An increased incidence

of myopathy has also been shown in patients with acute coronary syndrome treated with simvastatin (80mg daily) compared to placebo or simvastatin at lower dose regimens (20 mg daily) (de Lemos et al., 2004), but not for atorvastatin (80 mg daily) (LaRosa et al., 2005, Cannon et al., 2004), even though both drugs are lipophilic. Similarly, a greater incidence of statin related myopathy was attributed to atorvastatin (80 mg daily) compared to simvastatin (20 mg daily) (Pedersen et al., 2005) but this did not occur when atorvastatin (80 mg daily) was compared to either atorvastatin (10 mg daily) (LaRosa et al., 2005) or placebo (Amarenco et al., 2006).

Physicochemical properties of statins determine their bioavailability and thereby the risk of muscle toxicity. A statin's solubility in water affects its permeability across cell membranes of non-hepatic (including muscular) cells and its ability to cross the blood-brain barrier. Pravastatin, rosuvastatin and to some extent, fluvastatin, exhibit hydrophilic properties, whereas other statin molecules i.e. atorvastatin, simvastatin and lovastatin are lipophilic (Hamelin and Turgeon, 1998). The lower risk of myotoxicity associated with pravastatin therapy appears to be related to its decreased penetration of the lipid-rich membranes (Ziegler and Stunkel, 1992) and thus uptake by extra-hepatic tissues, presumably associated with the hydrophilicity of the molecule. Pravastatin is taken up by hepatic cells via the sodium-independent bile acid transporter, the OATP (Ziegler and Stunkel, 1992). Along with the sodium-dependent taurocholate co-transporting polypeptide, OATP also mediates the uptake of the hydrophilic rosuvastatin molecule by hepatocytes. Both *in vivo* (Nakahara et al., 1998) and *in vitro* (Gadbut et al., 1995) experiments are in agreement that lipophilic statins increase the risk of muscular adverse effects more than hydrophilic ones. However, the hydrophilicity of some statins has not been proven to offer clinically significant muscular protection (Bays, 2006). No clinical evidence exists that supports a direct association between the degree of lipophilicity and the myotoxic potential (Evans and Rees, 2002) since cases of rhabdomyolisis have also been attributed to hydrophilic statins. The susceptibility to statin myopathy may therefore be enhanced by factors that increase statin's concentration in intramuscular tissue including patient profile (**section 1.4.1**) and/or the presence of concomitant drugs (will be discussed in the next section).

1.4.3 Statin-drug interactions

The identification of other factors such as concurrent medications suggested that patient characteristics are not a sole determinant of the SRM. The SRM caused by statin-drug interactions, as a result of inhibition of and/or genetic polymorphisms of cytochrome P450 enzyme system or membrane transporters, have been described (Neuvonen et al., 2006).

There are basically three groups of drugs that may interact with statins causing SRM as shown in **table 1.4** below. Overall, there is no evidence to implicate whether statin therapy with other group of lipid lowering drugs such as fibrates, niacin and ezetimibe induce muscle toxicity (Chatzizisis et al., 2010). Although Shek and Ferrill (2001) have reported that there was 0.12% incidence of myotoxicity when statins are administered in combination with fibrate therapy, the incidence, however, is considerably low to conclude its relevance to the whole patient population.

Table 1.4 **Substances that may precipitate statin-induced myopathy** (adapted from Chatzizisis et al., 2010).

Interacting drugs/ co-medications	Postulated mechanism
Non-hypolipidaemic medicines	
Cyclosporin	CYP3A4 inhibitor
Macrolide antibacterials (erythromycin, clarithromycin)	CYP3A4 inhibitor
Azole antifungals (itraconazole, ketoconazole, fluconazole)	CYP3A4/CYP2C9 inhibitor
Calcium channel antagonists (diltiazem, verapamil)	CYP3A4 inhibitor
Nefazodone	
HIV protease inhibitors (ritonavir, nelfinavir, indinavir)	CYP3A4 inhibitor
Warfarin	CYP3A4/CYP2C9 inhibitor
Histamine H_2 receptor antagonists (cimetidine, ranitidine)	CYP2C9 inhibitor*
Omeprazole	CYP3A4 inhibitor
Amiodarone	CYP3A4 inhibitor
Hypolipidaemic medicines	
Fibrates (gemfibrozil > bezafibrate, clofibrate, fenofibrate)	Inhibition to statin glucoronidation (gemfibrozil only), activation of the peroxisome proliferator-activated family of nuclear receptors (interaction with fibrates)
Niacin	Unknown
Other substances	
Grapefruit juice	CYP3A4 inhibitor
Over-the-counter medications (Chinese red rice fungus)	Not available

*No particular clinical significance reported during the interaction with fluvastatin

Concurrent treatment with drugs which can affect the absorption, metabolism, excretion or protein binding of statins can increase the risk of myotoxicity (Bellosta et al., 2004). About 60% of cases of statin-related rhabdomyolysis are related to drug interactions (Kashani et al., 2006). Additionally, most of the reported statin-drug interactions are associated with the interference with hepatic metabolism of statins (Bottorff, 2006). For instance, simvastatin and lovastatin are metabolised by CYP3A4 and appear to be more susceptible to the inhibiting effect of other drugs which are substrates for the same enzyme such as cyclosporine or calcium channel antagonists as shown in the table (**Table 1.4)**. Similarly, the interaction between fluvastatin, which is metabolised by CYP2C9, and drugs that interfere with the CYP2C9 (e.g. Histamine H_2 receptor antagonists and azole antifungals) may be of clinical importance in SRM. However, rosuvastatin is minimally metabolised by CYP2C9 and therefore concomitantly administered drugs which interfere with this enzyme are of no consequence to its hepatic clearance.

Apart from the role played by the CYP enzymes, transporter-mediated drug-drug interactions (DDIs) may occur through inhibition or induction of transporter mediated influx or efflux. Neuvonen and colleagues (2006) have provided extensive review of clinically relevant drug interactions with lipid lowering drugs including statins that lead to altered pharmacology properties of the lipid lowering drugs (Neuvonen et al., 2006). Indeed, examples of DDIs involving drug transporters have been documented since more than five decades ago. These include; elevated plasma concentrations of digoxin and central opioid effects of loperamide when used concomitantly with MDR1 inhibitors (Di Rosa and Di Rosa, 2014, Kim et al., 1999), elevated serum levels of penicillin, angiotensin-converting enzymes (ACE) inhibitors and HIV antiviral drugs when they were co-administered with probenecid, inhibiting OAT-mediated secretion in the tubular kidney cells (Ayrton and Morgan, 2001, Burnell and Kirby, 1951). In the case of statins, there is accumulating evidence describing the interaction between SLCO1B1 and genes transcribing CYP metabolising enzymes. For example, it has been suggested that simvastatin is likely to be affected by the drug interaction as it is the most selective substrate for both SLCO1B1 and CYP3A4 genes, thus further suggesting gene-gene-drug interactions to illustrate possible clinical outcomes (Sadee, 2013).

1.5 Genetic risk factors of SRM

Genetic predisposing factors have been an active area of research in SRM for more than a decade. Recently, Maggo and colleagues (Maggo et al., 2011) have described the pharmacogenetic influence on SRM into two components; polymorphisms affecting genes involved in (a) statin pharmacokinetics (PK) and (b) statin pharmacodynamics (PD). Although a substantial number of studies have described the candidate genes involved in statin PDs (see **Table 1.5**), much of the reported adverse effects of statins were determined among candidate genes that were characterised by statin PKs (Shitara and Sugiyama, 2006). In general, the CYP metabolising enzymes and membrane drug transporters are the two major biological factors that determine PKs of statins. For lipophilic statins such as simvastatin and atorvastatin, which commonly undergo phase I oxidation, polymorphisms in CYP enzyme (typically CYP3A4, CYP2D6 and CYP3A5) can greatly affect PKs of the statin. Using the US Food and Drug Administration Adverse Event Reporting System database, the adverse events reporting rate and ratio (AERR) of rhabdomyolisis were compared for simvastatin (a statin 3A4 substrate) and pravastatin (a statin non-3A4 substrate) when each drug was coadministered with a CYP3A4 inhibitor. The study showed a six-fold increase in the AERR for simvastatin (with vs without a CYP3A4 inhibitor) and no increase in AERR for pravastatin (with vs without a CYP3A4 inhibitor) (Rowan et al., 2009). The hydrophilic statins, as they are not metabolised by the CYP enzymes, are eliminated largely as unchanged and therefore, polymorphisms in the CYP enzymes are not determinants in the side-effects of the hydrophilic statins.

Table 1.5 Summary of important genetic factors involved in PK/PD properties of statin metabolism underlying statin-related myopathy (adapted from Maggo et al., 2011).

Genetic factors that may alter PKs of statin metabolism	Genetic factors that may alter PDs of statin metabolism
Cytochrome P450 (CYP) 3A4	Adenosine Triphosphate-Binding Cassette Transporters and CYP7A1
CYP2D6	P-Glycoprotein (P-gp)
CYP3A5	Apolipoprotein E protein (Apo E)
Organic anion transporting polypeptides 1B1 (OATP1B1)	Cholesteryl ester transfer protein (CETP)
P-Glycoprotein (P-gp)	HMG-CoA Reductase Enzyme
	Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)
	Atrogin-1

Aside from the CYP enzyme system, hepatic membrane transporters, which include both uptake and efflux transporters, have been also considered to be determinants in the PKs of statins as well as their interactions with other drugs (Rodrigues, 2010). Until now, as stated earlier (**section 1.3.2**), common variants in SLCO1B1 (among the PKs factors) have been recognised as a major factor affecting statins exposure, as a result of reduced hepatic uptake of statins and consequently increased risk to muscle toxicity (Niemi et al., 2011). In a GWAS in patients with established myopathy and those with new-onset symptoms receiving simvastatin 80 mg daily, common variants in the SLCO1B1 gene were linked to a significant increased risk of myopathy, with 60% of all myopathy cases attributable to one specific common non-synonymous variant rs4149056 (521T>C). The odds ratio for myopathy was 4.5 (95% confidence interval, CI, 2.6 to 7.7) per copy of the C allele, and 16.9 (95% CI, 4.7 to 61.1) in CC as compared with TT homozygotes (Link et al., 2008). Notably, these associations were demonstrated among patients receiving high doses of simvastatin, indicating that genetics is a prominent factor in SRM and that genotyping of patients for the SLCO1B1 variants may help to improve the safety of statin therapy, particularly in patients receiving high-dose regimen and with coexisting risk factors.

On the other hand, genetic variants relevant to statin PDs could also be shown to influence statin efficacy. There are genes (refer **Table 1.5**) that code for proteins involved in the mechanism of action of statins, and therefore influence PD processes. This type of variation will not affect drug metabolism or transport, and will not impact on plasma or tissue levels of the drug. Take an Apo E variants for instance, it has been shown that Apo E variants were associated with variable response to statin treatment. Pharmacologically, Apo E binds to lipids and lipoprotein receptors, and modulates lipoprotein levels by influencing the clearance rate, lipophilic conversion as well as VLDL and TG production (Elghannam et al., 2000). A study comprising 328 males and females treated with 10 mg atorvastatin for one year indicated that males with Apo E2 variant allele showed significantly enhanced efficacy with respect to reductions in LDL-C (44%; p= 0.021), TC (34%; p = 0.033) and triglyceride levels (27%; p= 0.049) compared with patients with the E3 or E4 alleles (Pedro-Botet et al., 2001). Regarding fluvastatin therapy, the Apo E 3/3 genotype has been attributed to enhanced efficacy with respect to reductions in plasma TC (20.4% vs 15.4%; p= 0.01) and LDL-C (28.7% vs 22.7%; p= 0.03) compared with Apo E 3/4 or 4/4 genotypes (Ballantyne et al., 2000). Furthermore, patients with the Apo E 2/3 genotype had a greater increase in HDL levels (19.1% vs 4.3%; p= 0.002) in

response to fluvastatin therapy than those with genotypes 3/3, 3/4 and 4/4 (Ballantyne et al., 2000).

It is rather confusing on the group that P-gp (encoded through the ABCB1/MDR1 gene) might be presented on. The P-gp protein is another class of ATP-dependent efflux pump implicated in variable response to statins. One of the main features of this efflux pump is its broad substrate specificity and the ability to regulate the distribution, and hence bioavailability, of various drugs (Ambudkar et al., 2003, Fromm 2002). Therefore, it could be grouped among the PK components of statin metabolism. In fact, it has been reported that the AUC of simvastatin acid and atorvastatin was increased approximately 60 % in homozygous individuals for the c.1236T-c.2677T-c.3435T haplotype versus homozygous for the reference c.1236C-c.2677G-c.3435C haplotype of ABCB1 gene; no effect was seen on simvastatin lactone (Keskitalo et al., 2008). The P-gp transporter, on the other hand, has been also shown to influence PD of statins. The efflux transporter is known to transport many therapeutic drugs from the gastrointestinal tract to the circulation (antineoplastics, calcium channel antagonists, antibacterials, anticonvulsants and others) including statins (Schwab et al., 2003). Thus, inhibition of the P-gp transporter by statins may lead to reduction in efflux back into the gut during absorption. This increase in absorption of those drugs transported by this transporter results in reduced efficacy or ADRs due to altered drug translocation and accumulation (Leslie et al., 2005, Ambudkar et al., 2003).

With respect to genetic polymorphisms affecting lipid levels, allelic variants of the ABCB1 gene are known to affect the response to statin treatment (Kajinami et al., 2004b). 344 patients treated with atorvastatin 10 mg/day for 52 weeks were genotyped for two polymorphisms in the ABCB1 gene. The presence of the rs1045642 SNP, commonly referred to as the 3435T homozygous variant, in females was significantly associated with reduced efficacy with respect to LDL-C reduction and a moderate increase in high-density lipoprotein cholesterol (HDL-C) (Kajinami et al., 2004b). A further study by Fiegenbaum et al. (2005) incorporating 116 hypercholesterolaemic patients treated with simvastatin 20 mg per day for 6 months investigated the effects of genetic polymorphisms in the ABCB1, CYP3A5 and CYP3A4 genes and their effect on the response to statin treatment. Results indicated that patients who were carriers of the ABCB1 c.1236T variant allele (rs1128503) demonstrated significantly ($p = 0.042$) enhanced efficacy with respect to reduction in plasma TC (29%) and LDLC (39.6%) levels compared with

patients carrying the wild-type allele (24.2% and 33.8%, respectively). In this study, no significant associations with statin efficacy were related to polymorphisms in the CYP3A5 and CYP3A4 genes (Keskitalo et al., 2008).

There is also evidence that other potential important genetic predictors of SRM, other than that of involvement of PK and PD components as mentioned above, that may have predicted SRM cases. Recently, a SNP in glycine aminidotransferase (GATM) that encodes the rate-limiting step in creatine synthesis i.e., rs9806699 was found to be associated with incidence of SRM in two separate populations with meta-analysis odds ratio of 0.60 (Mangravite et al., 2013). Thus this SNP was earlier regarded to be among promising gene candidates in SRM. The findings from a subsequent gene association study using a large, multicenter case-control study, however, did not replicate the findings by those of Mangravite et al. (2013). The GATM rs9806699 allele/genotype frequencies were found to be similar in statin myopathy cases and controls with unadjusted odds ratio for the A allele for any mild or severe statin myopathy was 1.14 (0.82 – 1.61; p=0.437) (Luzum et al., 2015), which was consistent to that found by two earlier studies (Carr et al., 2014, Floyd et al., 2014). There is also evidence of the involvement of mitochondrial stress and cellular reactive oxygen species (ROS) formation in SRM based on data from both human and animal studies (Kwak et al., 2012, Bouitbir et al., 2011). As reported by Larsen and colleagues (Larsen et al., 2013), the apparent 60% loss of glutathione peroxidase (GPx), an antioxidant enzyme, in patients chronically treated with simvastatin, may explain in part, the contribution of oxidative stress in decreased mitochondrial function and glucose intolerance seen in simvastatin related-skeletal muscle effects. This suggests polymorphism in genes transcribing antioxidant enzymes including GPx, catalase and members of the superoxide dismutase could to a degree, contribute to SRM along with other coexisting genetic polymorphisms. Finally, although the involvement of immune function is not widely established as an underlying cause for statin adverse reactions, the HLA class II allele, DRB1*11:01 was identified as a strong genetic risk factor for the development of one specific statin myopathy, anti-HMGCR-associated necrotizing immune myopathy (Mammen et al., 2012).

1.6 Population Candidate Gene Association Studies

Genetic association studies test for a correlation between disease status and genetic variation to identify candidate genes or genome regions that contribute to a specific disease. The studies are major tools for identifying genes conferring susceptibility to complex disorders which mainly involve both genetic and environmental factors, and SRM is one such example. There are two approaches used in the genetic association studies; family-based design and population-based design. The advantages and pitfalls of these two designs have been described previously (Daly and Day, 2001). A typical population-based design is the case-control study, which is commonly designed retrospectively in genetic studies, comparing genetically unrelated individuals with the disease with appropriate controls (either community or clinical-matched controls). A large-scale for case-control design in genetic association studies has been featured in genome-wide association studies (GWAS) to detect associations using 500,000 to more than a million genetic markers.

1.6.1 Single marker analysis in case-control design

In genetic association studies, single marker analysis for both matched and unmatched case-control study has been described (Zheng et al., 2012). Simulation methods for matched case-control studies is rather more stringent than that of an unmatched method, as the purpose for the matched methods is often used to control for confounding variables and a known population (Zheng et al., 2012). Common test statistics for genetic association covered in the unmatched case-control method normally include genotype-based tests (i.e., Pearson's chi-squared test, the Cochran-Armitage trend test, and the likelihood-ratio test) as described by Zheng and colleagues (2012). In genetic association studies, the data for case-control studies may be analysed assuming these pre-specified genetic models - dominant, recessive, multiplicative and additive genetic model. Please refer to **Appendix A** for further detail.

1.6.2 Candidate SNPs of known functional significance

There are factors to take into account during performing case-control studies. These factors include study design, methods for recruitment of cases and controls, functional significance of the selected polymorphisms for study and statistical analysis of data to

ensure that only genuine associations are detected (Daly and Day, 2001). Selection of SNPs with functional effects and candidate genes of disease pathway were considered the first step in candidate gene association studies. This method offers considerable advantages in terms of detecting disease associated genes, rather than randomly selected polymorphisms in particular genes (Risch, 2000). The availability of detailed information on functional significance before the initiation of an association study should help to avoid reporting of spurious associations (Roses, 2000). Functional effects of polymorphisms, however, are complex to understand, and it is important that the overall effects of possession of a particular haplotype, which may include tagging SNPs of non-functional variants, be considered rather than the functional effects of a single polymorphism (Daly and Day, 2001). With the development of haplotype-tagging system, which is used to detect tag-SNP (will be described in s**ection 1.6.3**) in the International HapMap project, non-functional variants found in close proximity to functional markers or in linkage disequilibrium (LD) with other true functional polymorphisms, are also now feasible for the candidate gene studies (Stram, 2004).

With the introduction of GWAS studies, certain genes in the candidate gene approach can be obtained from the findings, which then lead to more significant meaningful markers. As the GWAS manage to scan a large set of genetic variants and thus to identify associations with a particular trait or disease of which the traditional candidate gene method cannot do, the impact of candidate genes (resulted from the GWAS) on later studies cannot be denied. So far, only one GWAS has been established in the field of statin myopathy (Link et al., 2008) and the study has given a good example on how GWAS findings has produced subsequent successful replication studies. The GWAS, conducted in 2008, was carried out using a matched case-control study (n_{cases} = 85, $n_{controls}$ = 90) from the 12,000-patient-strong Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) trial. The findings in the GWAS presented how the knowledge of a linked polymorphism i.e., a tag-SNP, in a synonymous SNP (rs4363657) in SLCO1B1 gene will be useful in determining coding SNP (i.e., rs4149056) which in complete LD with the rs4363657 (r^2 = 0.97) thus indirectly interpreted the functional significance of the genetic marker.

1.6.3 Candidate SNPs in tagging SNPs

With the completion of the Human Genome Project (Venter et al., 2001) and the HapMap Project (Altshuler et al., 2010) increasing knowledge of the whole human genome and information about the location and function of genes, has prompted the suggestion of genetic association studies by selecting (presumably small) number of tagging SNPs (tag SNPs) for complete analysis of the haplotype structure of the human genome (Zhang et al., 2004). Tag SNPs are a reduced set of SNPs that capture much of the LD region and they can be used in whole-genome SNP association studies to reduce the number of SNPs needed to detect LD-based association between a trait of interest and a region in genome. Therefore, the use of tag SNPs, either by haplotype block-dependent or block-free methods (Zhang and Sun, 2007), in association studies can significantly reduce the effort put on genotyping of the whole SNPs in a gene.

Unlike haplotype block-dependent methods, in the block-free methods, tag SNPs will be selected according to LD pattern (Nicolas et al., 2006, Carlson et al., 2004, Lin and Altman, 2004) or by using power computations (Byng et al., 2003, Cousin et al., 2003). There were three independent steps in the haplotype block-free selection of tagging SNPs; (i) identifying genomic segments where the tag SNP will be performed; (ii) defining a measure to quantify how well a set of tag SNPs can predict all observed and/or unobserved SNPs; (iii) searching a minimum set of tag SNPs that meets a desired threshold (Halldorsson et al., 2004). Although it has been argued that the use of tag SNPs, instead of all the SNPs, resulted in the loss of power in association studies, the loss of power was moderate (Zhang et al., 2002). The tag SNPs, however, are not sole determinant in this case, as many other factors such as sample size, number of SNPs used, MAF value, fraction of missing data and genotyping errors, has been found to affect the power of association studies (Zhang et al., 2004).

1.7 Research Plan

The work presented in this thesis is based on both cellular and clinical studies with the aim being that the findings of cellular work would result in some candidate SNPs which could then be tested in a clinical study. Since muscle toxicity is a common side-effect of statins, investigation of the effect of statins on transporters located in muscle cell membrane can be important in elucidating SRM. While there is evidence for the

involvement of OATP and MRP in transport of statins in striated muscle (Knauer et al., 2010), there is no information available on the involvement of MCTs, which are abundantly expressed in the skeletal muscle, and their polymorphisms in relation to SRM. We hypothesised that SRM could be promoted by the increased local uptake of statins and/or accumulation of endogenous monocarboxylate metabolites (e.g. lactate) in skeletal muscle which mainly involve MCTs, possibly MCT1 and MCT4. Since the interplay between statin uptake versus efflux transporters modulates the response to skeletal muscle statin exposure, it is also of interest to determine statin impact on efflux transporter, possibly members of the MRP family.

The statin transporters information obtained from the cellular work could be tested for genotype-phenotype relationship in SRM. This, along with a selection of other candidate genes with known clinical significance for statin efficacy and side-effects, will be studied in clinical setting using a case-control study. There has not yet been a genetic association study carried out to identify the association between SNPs in MCTs and SRM cases. Therefore, the general aim of this study is to gain better understanding into the risk factors that are responsible for the inter-individual variability to statin-induced muscle toxicity, by analysing data from both the preclinical and clinical study.

Over the three years of my research, the following studies were planned to be undertaken:

1. To assess statin impact on functional activity of MCT uptake transporters and efflux transporters, MRPs in particular, in a high-expression-MCT1 and -MRPs cell line model, HK-2 (a proximal tubule cells) cells.

2. To replicate the same functional studies in a model of skeletal muscle cells from both rat and human.

3. To evaluate the association between patient demographics (age, sex or body mass index), genetics and other contributory factors to the occurrence of SRM in a retrospective case-control study.

Chapter 2. Effects of Statins on Functional Expression of Monocarboxylate Transporters (MCTs)

2.1 Introduction

There are numerous factors that influence statin-related myotoxicity (SRM), including patient characteristics, genetics, drug-drug interactions and statin dose as described in Chapter 1. Genetic variability of membrane transporters in hepatocytes has been shown to play a crucial role in determining statin plasma concentrations and the subsequent risk in developing myopathy. Although it has been established and recognised that elevated plasma statin concentration increases the risk of muscle toxicity (Huerta-Alardin et al., 2005, Jones and Davidson, 2005, Ballantyne et al., 2003), the aetiology of statin-induced muscle toxicity is not well understood.

It has been shown that exposure to lactate, a monocarboxylate, was found to be associated with the generation of reactive oxygen species (ROS) and the up-regulation of genes related to mitochondrial lactate oxidation complex in both *in vitro* and *in vivo* study using rat skeletal muscle cells (Bouitbir et al., 2011, Hashimoto et al., 2007). In muscle, lactate has been transported by monocarboxylate transportres (MCTs), possibly MCT1 and MCT4 since they are highly expressed in the striated muscle (Adijanto and Philp, 2012, Halestrap and Price, 1999). Physiologically, the role of MCTs in skeletal muscle is undeniable since the organ is the major site of lactate production and removal in the body. So far, there has been no study to investigate the association of the MCTs with the risk of SRM. Substrates for MCTs have not been limited to endogenous metabolites but also xenobiotics such as statins, gamma-hydroxybutyrate (GHB) and valproic acid (Morse et al., 2014, Liu et al., 2013, Kobayashi et al., 2006, Sirvent et al., 2005).

Our group has previously shown that HK-2 cells express MCT1 at the mRNA and functional levels (Jenkinson et al., 2012). Since statin-related side-effects are associated with muscle cells, I investigated statin effects on the expression of MCTs in two *in vitro* muscle models, namely the L6 rat skeletal muscle cells and the cells originated from primary human muscle cells. A number of studies have characterised MCT functions in the L6 cells (Hashimoto et al., 2007, Hashimoto et al., 2006, Kobayashi et al., 2004). However, there is no conclusive evidence that statins affect MCT function in muscle cells. This study thus aims to compare the effect of statins in the functional expression of MCT in these three cell lines. We hypothesised that statin-induced muscle toxicity could be promoted by an increase in the local uptake of the drug by striated muscle and/or accumulation of endogenous metabolites such as monocarboxylates lactate. I thus

investigated the effects of statins on functional expression of MCTs which could act as mediators of statin transport in muscle cells. It is possible that the inhibition of the transporter functions by statins could advocate myotoxicity due to intracellular lactate and/or statin accumulation.

2.2 Materials and Methods

2.2.1 Materials and reagents

RT^2 Profiler™ Human Drug Transporter PCR array (Catalogue no: PAHS-070Z), Rat Drug Transporter PCR array (PARN-070Z) and reagents were purchased from Qiagen Ltd (Crawley, UK). Simvastatin, atorvastatin, pravastatin, and rosuvastatin were gifts from AstraZeneca (Alderley Park, Cheshire, UK). [$^{2\text{-}3}$H]-DL-lactate (at activity of 20 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). SV Total RNA Isolation System was purchased from Promega (Southampton, UK). SYBR Green Dye Master Mix for real-time polymerase chain reaction was purchased from Roche Applied Sciences (Burgess Hill, UK). 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulpahte (PMS) were obtained from Promega (Southampton, UK). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (Dorset, UK) and were of the highest quality available.

2.2.2 Cell culture and maintenance

Three cell lines were used in this project; human proximal tubule cells (HK-2), rat skeletal muscle cells (L-6) and human skeletal muscle myotubes. The cells were cultured and maintained as described as follow;

a. HK-2 cells

The HK-2 (ATCC® number: CRL-2190™**)** cell line was a gift from Professor John Kirby, Newcastle University. The cells are derived from normal human epithelial renal proximal tubule and immortalized with a recombinant virus (Human Papilloma Virus 16, HPV 16) containing E6 and E7 genes. The cells were used up to 20 passages from the initial culture and cultured in Dulbecco's modified Eagle's medium (DMEM) high

glucose/Ham-F12 medium at a 1:1 ratio, supplemented with 200 units/ml penicillin, 200 µg/ml streptomycin, 5 mM L-gluatmine and 10% fetal bovine serum.

b. L6 rat muscle cells

The L6 rat muscle cell line (ATCC® number: CRL-1458™), supplied at myoblast stage, was kindly provided by Dr Audrey Brown of Newcastle University. The cell line was established from embryonic rat thigh muscle (Yaffe, 1968). L6 exists as myoblasts when cultured in maintenance culture medium, which comprises high glucose DMEM supplemented with 10 % foetal calf serum, 200 units/ml penicillin and 200 µg/ml streptomycin, until 70-80% confluency. The cells are then introduced to differentiation medium (high glucose DMEM supplemented with 2 % horse serum, 200 units/ml penicillin and 200 µg/ml streptomycin), upon which the cells differentiate into myotubes until confluent (**Figure 2.1**). L6 cells used in this project were within passage number 11 and 30.

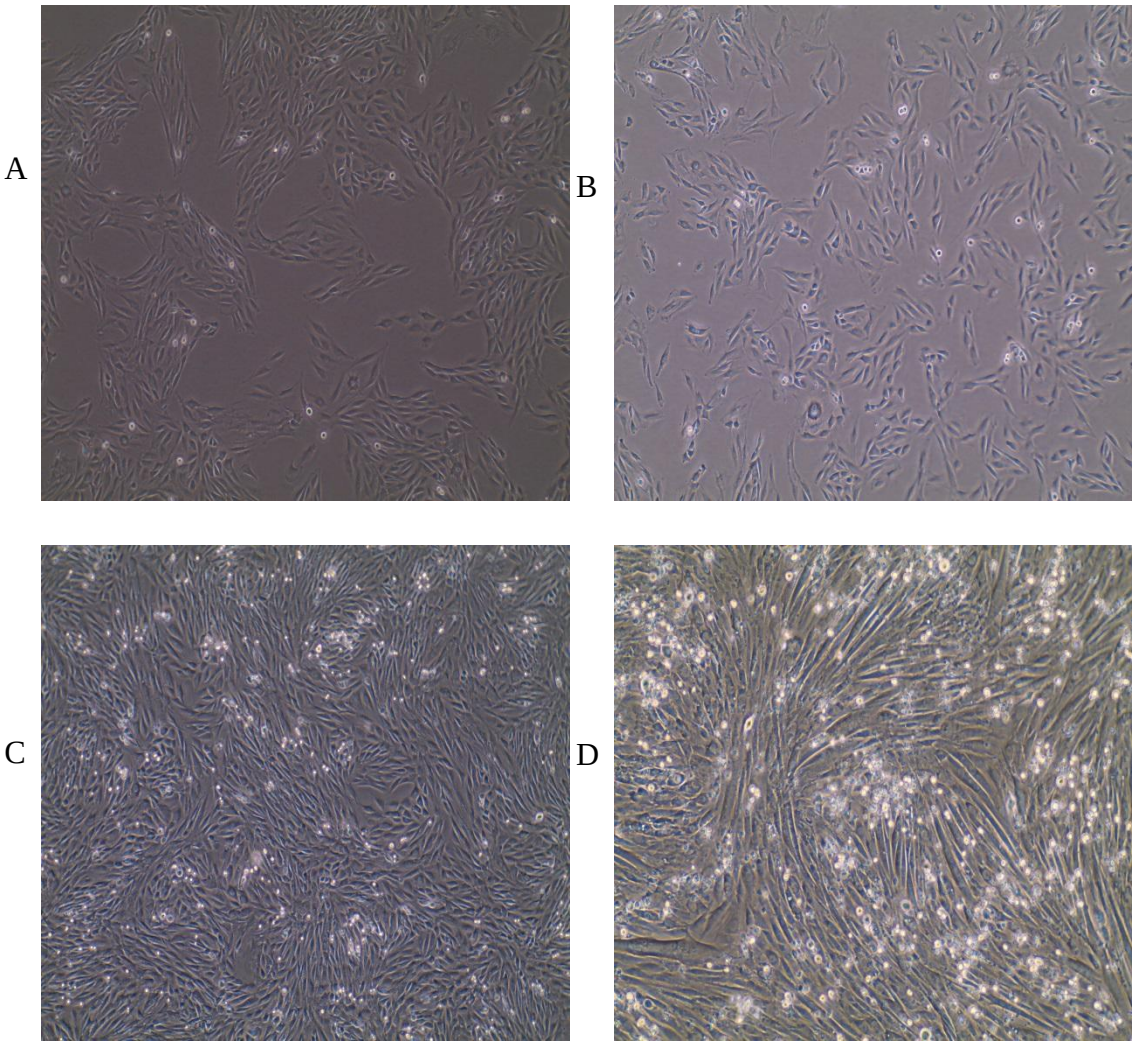


Figure 2.1 Visualization of various stageof L6 cell line cultured in a 24-well plate. Initial cell density at day 0 was 1 x 10^5 cells/mL and cultured with 500 µl growth media. Cells were then viewed; at myoblast stage on Day 1 (A), myoblast stage on Day 2 (B), and confluence on Day 3 (C). Subsequent cell maintenance with differentiation media transformed the myoblast into myotube structure on day 6 or 7 after differentiation (D) (40x magnification). Single representative field of view randomly selected.

c. Cultured human muscle cells

Human muscle cells used in this project was supplied by Dr Audrey Brown of Newcastle University. These cells were sourced from healthy subjects with no family history of type 2 diabetes. The subjects were recruited previously for another study by Brown and colleagues (Brown et al., 2007) for which full ethical approval was held. The cells were obtained from the vastus lateralis and satellite cells isolated as described by Blau and Webster (1981) (Blau and Webster, 1981). For this study, the cells, initially obtained as myoblasts, were used within passage 4 and 8 as previously described for cultured human skeletal muscle cells (Brown et al., 2008). The myoblasts were cultured in Ham's F10 medium supplemented with 20% (v/v) FBS, 2% (v/v) chick embryo extract, 100 units/ml penicillin and 100 μg/ml streptomycin, for approximately 5 to 7 days to reach 70-80 % confluency. The cells were subsequently differentiated into myotubes for another 6 or 7 days (**Figure 2.2**) by the introduction of minimum essential medium (MEM) supplemented with L-glutamine and 2% (v/v) FBS. All experiments were performed using differentiated myotubes from three different subjects.

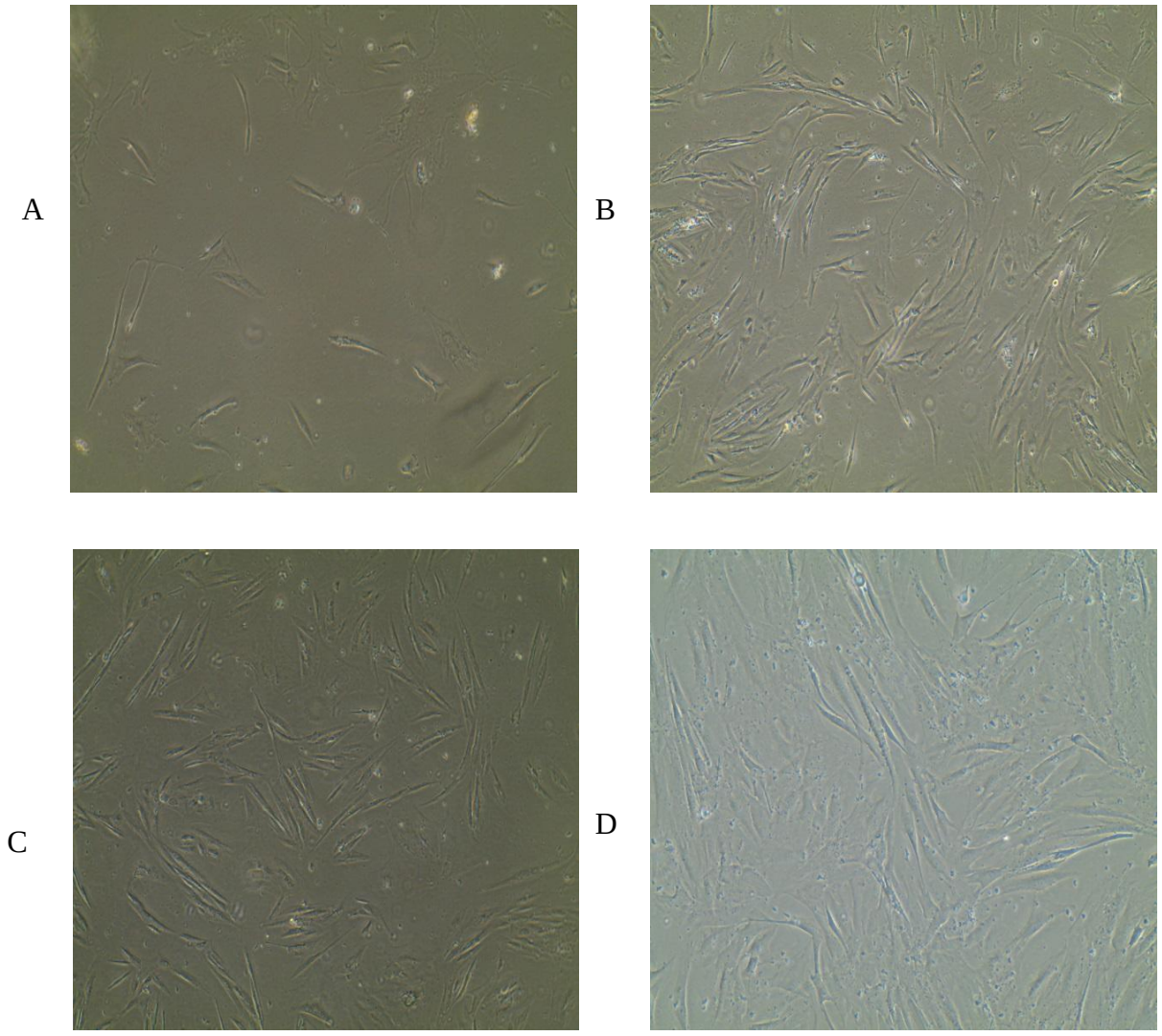


Figure 2.2 Visualization of various stages of human muscle cell line cultured in a 24-well plate. Initial cell density at day 0 was 1 x 10^5 cells/mL and cultured with 500µl growth media. Cells were then viewed; at myoblast stage on Day 3 (A) , myoblast stage on Day 6 (B), and the appearance of the myoblast on day 9 (C) remained the same as Day 6 (B). Subsequent cell maintenance with differentiation media transformed the myoblast into myotube structure on day 6 or 7 after differentiation (D) (40x magnification). Single representative field of view randomly selected.

2.2.3 Routine passage and maintenance

All cell types were routinely grown in 75 cm^2 (T75) flasks, each containing 15 ml of growth medium and incubated in a humidified incubator at 37°C and 5% CO_2. Cells were passaged when they reached 80-90 % confluency. Passage of cells involved removing old medium and the adhered cell monolayer washed twice with 10 ml PBS before introduction of 0.25% trypsin/EDTA for 5 minutes at 37 °C to dislodge the cells. Equal volume of complete medium was added to deactivate the trypsin and the cell suspension was then centrifuged at 1500 rpm for 3 minutes to remove residual trypsin. Fresh medium was added to the cell pellet and resuspended by passing the mixture through a wide-bore needle. Cell count was performed using Cellometer Automated Cell Counter (Nexcelom Bioscieces, Lawrence, MA) before downstream applications.

1 x 10^6 passaged cells were transfer to a clean T75 cell culture flask for further propagation and cultured as described above. Passaged HK-2 cells were either seeded onto 12-well plates at a density of 100,000 cells/well or 96-well plates at a density of 20,000 cells/well. The cells were cultured for 2 and 3 days using 96-well plates and 12-well plates, respectively, before use in experiments. The other cell types (i.e., L6 rat and human muscle cells) were seeded onto 24-well plates at 50,000 cells/well or 96-well plates at 20,000 cells/well. They were cultured for 5 to 7 days to allow cells to reach 70-80 % confluency. After which they were differentiated by changing the culture medium to the differentiation medium. Differentiation media were changed every two days.

Routinely, 1 x 10^6 cells were cryopreserved in liquid nitrogen to maintain low passage number. This involved suspending the cells in 1 ml of freezing medium (culture medium with 10 % fetal calf serum and 10 % DMSO) before gradual temperature drop (-1 ºC/minute in nitrogen vapour) and stored in liquid nitrogen at temperature of -135 ºC. The cryopreserved cells were thawed quickly in a 37 ºC water bath and supplemented with warm fresh medium, and cultured as described above.

2.2.4 Assessment of cell viability by MTS assay

MTS assay was used to assess the viability of all cell types used in this project. Medium of cells seeded on 96-well plates were aspirated gently and the adhered cells washed three times with warm modified-Krebs buffer (137 mM NaCl, 5.4 mM KCl, 0.3 mM NaH_2PO4, 1 mM $MgSO_4$, 0.3 mM K_2PO_4, 2.8 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, to pH 7.4 with Tris base). The cells were then equilibrated with 100 µl modified-Krebs buffer for several minutes before 20 µl MTS/PMS mixture was added. The plate was incubated at 37 °C in the absence of light and absorbance of the formazan produced was measured at 490 nm after 4 hours using FLUOstar Omega Microplate Reader (BMG LabTech, Germany).

2.2.5 RNA extraction and analysis

a. RNA extraction

Cell monolayers for RNA extraction were washed twice with ice-cold PBS and scraped into PBS and pelleted by centrifugation for 5 min at 1500 rpm. Total RNA was then isolated from the cells using SV Total RNA Isolation System (Promega) as per manufacturer's instructions. RNA yield and quality were then quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples included had a RIN (RNA integrity number) greater than 8. The RNA was then stored at -80°C for further use.

b. Quantitative real-time PCR arrays (qPCR)

Th real-time PCR (or qPCR) is the method of choice to quantify expressed genes of interest at the RNA level. The qPCR has become a well-established procedures for quantifying levels of gene expression, as well as gene rearrangements, amplifications, deletions or point mutations. Compared with conventionally performed semi-quantitative end point PCR (reverse transcription-PCR, or abbreviated as RT-PCR), the qPCR has its own advantages because of its high sensitivity, high specificity, good reproducibility and wide dynamic quantification range (Dorak, 2006).

There are three basic metadologies commonly used in the detection of RNA or DNA targets by the qPCR method and all of them utilise fluorescent dyes. The simplest assay system involves the incorporation of a free dye i.e., SYBR® Green I (EvaGreen™ and

BOXTO are the new free dyes that has been presented for use in qPCR) into the newly formed double-stranded DNA product. Fluorescence is emitted proportionally to the amount of double- stranded DNA. As described by Tevfik (2006), there are advantanges and disadvantages for the SYBR® Green-based detection method over the other assays in qPCR such Taqman-based detection assay (refer **section 4.2.7b**) in term of specificity and sensitivity. However, as required for both methods, optimal design of the PCR primers is essential for accuracy and specificity. For assays using SYBR® Green I dye, detection is based on the binding of the dye into double-stranded PCR products (as it accumulates during PCR), which is a sequence-independent process. While this assay is cheaper than the specific probe-based assay i.e., Taqman® assay (it uses a fluorogenic probe specific to target gene), it loses the additional level of specificity introduced by the hybridisation of a specific fluorescent Taqman® probe to the PCR product. The sensitivity of detection with SYBR® Green may therefore be compromised by the lack of specificity of the primers, primer concentration and the formation of secondary structures during PCR such as primer-dimers. The 5' nuclease assay using Taqman® probes would also be compromised by the lack of primer specificity and limiting primer concentration, and although these are not detected by the Taqman® probe, they alter the amplification efficiency of the PCR reaction (Dorak, 2006).

The quantitative endpoint for qPCR is the threshold cycle, C_T, or also known as crossing point (C_p). The C_T is defined as the PCR cycle at which the intersection between an amplification curve and a threshold line occurs when the ΔRn (Rn is the reporter signal normalized to the fluorescence signal of reference dye) is plotted against PCR cycle number values. By presenting data as the C_T, it can be assured that the PCR is in the exponential phase of amplification and the value is inversely related to the amount of nucleic acid present. There are two methods of presenting the quantitatitative measurement of gene expression i.e., absolute and relative quantification, as described previously (Schmittgen and Livak 2008). Unlike the absolute quantification method, the relative gene expression, which it is the preferred method of choice for this study to measure of fold change in mRNA level of a gene due to a treatment, presents the data of the gene of interest relative to some calibrator or internal control gene (refer **Appendix B1**). This is represented by the equation $2^{-\Delta\Delta C_T}$ (Livak and Schmittgene, 2001). This is an alternative method of presenting the qPCR data and thus proper validation of the internal control, or so-called housekeeping gene or lineage specific gene, is critical. The internal control gene should not change under experimental conditions (e.g. treated versus

untreated or normal versus diseased state). Validation of the internal control should be done prior to quantifying the gene of interest. Methodology to validate internal control genes has been described by Schmittgen and Livak (2008) and a hypothetical example to determine whether a gene is suitable for use as an internal control in a drug treatment experiment is presented in **Appendix B2.**

Using qPCR array plates from Qiagen Ltd (Crawley, UK), analysis of a panel of 84 drug transporter genes in the three cell lines studied (**section 2.2.2**) was carried out. The qPCR arrays are set of optimised real-time PCR primer assays on 96-well plate disc for pathway or diseased focused genes as well as appropriate RNA quality controls. The manufacturer's instructions were followed for the assay. Briefly, 1.5 µg of total RNA samples from L6 rat and human muscle cells were reverse transcribed to cDNA using RT^2 first strand synthesis kit supplied by the PCR array. SABiosciences RT^2 2x SYBR Green Master Mix (SABioscience) was added to the diluted cDNA. 25 µl of the mixture was then aliquoted into each of the well of the array and qPCR carried out using Lightcycler 480 Real-Time PCR System (Roche Applied Science, Burgess Hill, UK) with the following programme: 95 °C, 10 minutes for 1 cycle followed by 45 cycles of 95 °C for 15 seconds, 60 °C for 1 minutes. There are five housekeeping genes (Actin, Beta-2 microglobulin, Hypoxanthine phosphoribosyltransferase 1, Lactate dehydrogenase A and Ribosomal protein) were used to normalise the expression levels using the RT^2 Profiler^{TM} array plate. Both the C_T and $2^{-\Delta\Delta C_T}$ values can be automatically retrieved and analysed using SABioscience web-based portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php.

2.2.6 Functional assay of monocarboxylate transporters (MCTs)

a. ^{3}H-DL-lactate uptake assay

Lactate uptake by HK-2, human cells, and L6 cells seeded on 12-well or 24-well plates in the presence of statins were carried out. Cell culture medium was aspirated off and adhered cells washed with pre-warmed modified-Krebs buffer three times. The cells were equilibrated with appropriate buffers for 20 minutes at 37 °C. Sodium-free Krebs buffer was made by replacing sodium chloride with choline chloride. Uptake was initiated by replacing the modified-Krebs buffer with buffer containing ^{3}H-labelled DL-Lactate (1 µCi/ml). After a pre-determined incubation period, the uptake was terminated by washing cells three times with ice-cold Krebs buffer. To determine whether inhibitors inhibit the

uptake function, cells were incubated with the ^{3}H-DL-lactate in the presence or absence of the inhibitors. Initial binding of radio-labelled compound to the surface of the cells was determined by measuring uptake at 0 min. The cell monolayers from each well were then solubilised with 0.5 ml of 0.05% (w/v) sodium dodecyl sulphate (SDS) and the cell lysates were transferred into individual scintillation vials (Meridian, Surrey, UK). Following addition of 1 ml Optiphase HiSafe scintillation cocktail (PerkinElmer, Beaconsfield, UK), samples were vortexed and the associated radioactivity determined using an LS6500 liquid scintillation counter (Beckman Coulter, High Wycombe, UK).

b. Calculation of radiolabelled substrate uptake

The amount of DL-lactate accumulated by the cell monolayer was determined using **Equation 2.1** below:

Equation 2.1

$$\text{Uptake (pmol)} = \frac{\text{Monolayer DPM x M}}{(\text{Standard DPM x 30})} \div \text{surface area (cm}^2\text{)}$$

Monolayer DPM (disintegrations per minute) is the amount of radioactivity accumulated by the cell monolayer. Standard DPM is the total radioactivity available to the monolayer, calculated from the average of three 10 µl samples of the transport solution. In the equation, in order to calculate the total DPM available to the cell monolayer, the Standard DPM is multiplied by 30 because the monolayer was bathed in 300 µl volume. M is the total amount of DL-lactate (in moles) available for uptake. To take into account the approximate growth area (i.e., 1.9 cm^2 and 3.8 cm^2 for 24-well plate, 12-well plate, respectively), the values calculated by **equation 2.1** are divided by the indicated surface area of the monolayer. Results are, therefore, are expressed as the amount of lactate accumulated by the monolayer, per cm^2, over time (pmol/cm^2/min). An example calculation for the ^{3}H-DL-lactate uptake from a monolayer of 24-well plate is shown in **Appendix C1**.

c. Measurement of K_m and V_{max} values

Calculation of the substrate concentration that yield half-maximal response, otherwise known as the Michaelis-Menten kinetic (K_m), and the maximal response (V_{max}) to describe the radiolabelled uptake kinetics was performed by GraphPad Prism software version 4 (GraphPad Software Inc. San Diego, CA, USA).

In order to show the potency of inhibitor to inhibit transporter-mediated substrate uptake, an IC_{50} value (the concentration of competitor required to inhibit substrate uptake by 50% of the maximum, and therefore cause 50% reduced uptake) was used to characterise the inhibition of uptake kinetics. For the purpose of this study, all IC_{50} values were derived from their respective K_m. The inhibition constant for the inhibitor (K_i) was calculated using **Equation 2.2** as described by Cheng and Prusoff (Cheng and Prusoff, 1973). K_i values account for the differences in the affinity of individual transporter, and for differences in probe substrate concentration used. The lower the K_i, as with the IC_{50} value, the more effective the inhibitor binds to a binding site and in this case, the membrane transporter, while the higher the K_i, the less effective the inhibitor binds to the transporter.

Equation 2.2

$$K_i = \frac{IC_{50}}{1 + ([ligand]) / K_m)}$$

where K_i is the binding affinity of the inhibitor, IC_{50} is the functional strength of the inhibitor shown by the concentration of inhibitor (for example, phloretin or statins) required to inhibit the increase in uptake activity by 50%, [ligand] is the fixed substrate concentration and the K_m value is the concentration of substrate for radioligand required to produce 50% maximal increase in uptake transporter activity.

d. ^{3}H-DL-lactate efflux assay

The efflux of radiolabelled substrate was used to functionally measure the efflux expression of MCT. Briefly, cells were pre-incubated with 300 µl of Krebs containing radiolabelled DL-lactate (50 µM) for 60-90 minutes at pH 6.0, 37 °C to ensure cells were loaded with radiolabelled substrate. After incubation, cells were then washed with ice-cold Krebs at pH 7.4 three times. To initiate lactate efflux, cell monolayers were

incubated with 300 µl pre-warmed ^{3}H-DL-lactate-free Krebs buffer at pH 6.0 with and without 500 µM inhibitor. After several time intervals, the cell monolayers were solubilised in 0.5 mL of 0.05 % SDS and transferred to scintillation vials. The radioactivity was measured as described earlier. Fractional efflux rate (% of radiolabelled lactate efflux every 2 min intervals) was calculated according to the **Equation 2.3** below (refer **Appendix C2** for an example of calculation) and the magnitude of MCT-mediated ^{3}H-DL-Lactate efflux in Krebs-inhibitor solution was compared to that of control with Krebs buffer only.

Equation 2.3

$$\text{Fractional efflux rate} = \left(\frac{\text{Amount of effluxed lactate}}{\sum \text{Remains of intracellular radioactivity}} \right) \times 100\%$$

2.2.7 Statistical analysis

All statistical analysis on data was performed using GraphPad Prism software version 4 (GraphPad Software Inc. San Diego, CA, USA). Statistical difference of continuous measurements was tested using Student's unpaired t-test (two groups) or a One-Way ANOVA (mean values for three groups or more) with Dunnett's post-hoc test as appropriate. A value of $p < 0.05$ was considered statistically significant. Unless otherwise stated, results are expressed as the mean ± SEM from separate experiments performed on separate days.

2.3 Results

2.3.1 Expression of MCT uptake transporter in HK-2 cells

Relative mRNA expression of key uptake transporters

The expression of a series of uptake transporters in HK- cells was carried out using qPCR array (catalogue no: PAHS 070Z). It was found that SLC16A1 (MCT1) mRNA was most abundantly expressed in HK-2 cells; however, no mRNA expression of SLC16A2 (MCT2) and SLC16A3 (MCT4) was detected in this cell line (data not shown in **figure 2.3**). Other SLC and SLCO groups of uptake transporters such as SLC22A2 (OCT2), SLCO2A1 (OATP2A1), SLCO3A1 (OATP3A1) and SLCO4A1 (OATP4A1) were also expressed at relatively lower concentrations. The pattern of expression of these uptake transporters is summarized in **figure 2.3**.

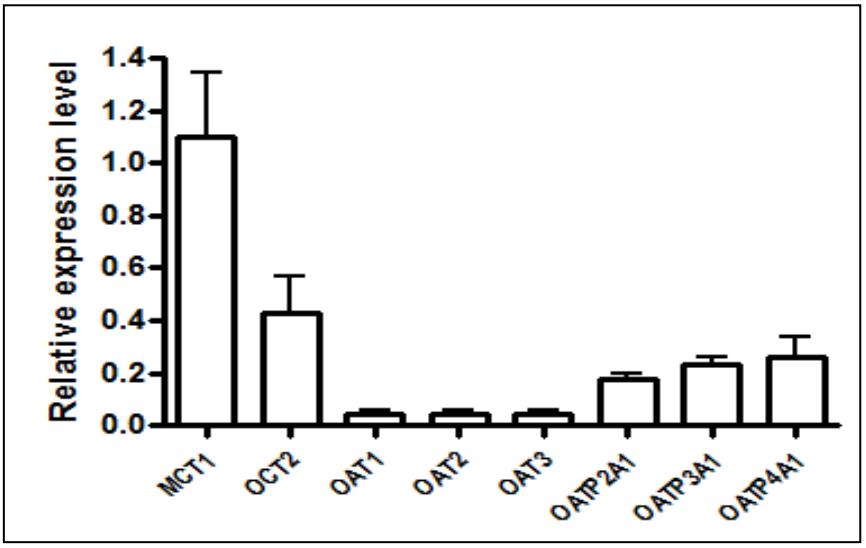


Figure 2.3 Relative gene expression levels of key uptake transporters in HK-2 cells. The relative gene expression level was analysed by standard RT² Profiler PCR Array data analysis from SABiosciences website. The expression levels were calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to the geometric mean of five housekeeping genes provided by the array. Error bars represent the mean ± SEM of three independent assays of three separate cultures of HK-2 cells. MCT, monocarboxylate transporter; OCT, organic cation transporter; OAT, organic anion transporter; OATP, organic anion transporting polypeptide.

Functional expression of MCT uptake transporter

Functional expression of MCT uptake transporter was determined by ^{3}H-DL-lactate uptake in the HK-2 cells. The kinetics of 3H-DL-lactate uptake by the cells is shown in **figure 2.4**. The uptake of DL-lactate (1 μCi/mL) was linear up to 15 minutes incubation time. The uptake of lactate was found to be saturable and concentration-dependent over the range concentration of 0.1 mM to 10 mM. A nonlinear regression analysis with a simple Michaelis-Menten equation gave a K_m value of 4.21± 1.0 mM (95% CI: 2.18 - 6.24). Importantly the uptake of lactate was significantly ($P<0.0001$) higher at a more acidic extracellular pH (uptake at pH 5.5 > pH 7.4). In addition the uptake of lactate was markedly Na^+-dependent. This result is not consistent with the characteristics of MCTs which are Na^+-independent transporters. It may reflect the expression of sodium-coupled monocarboxylate transporter (SMCT) and was Na^+-dependent.

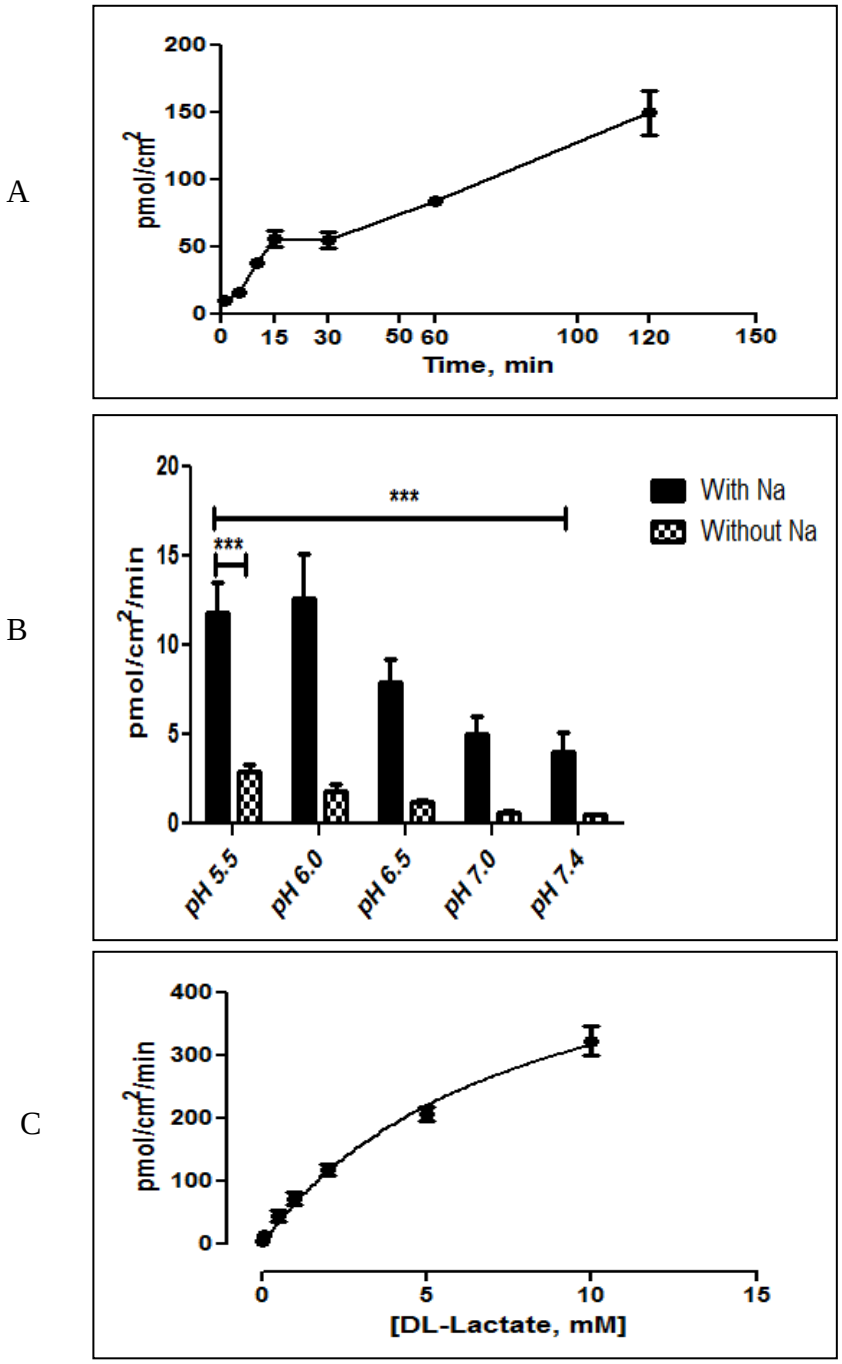


Figure 2.4 DL-Lactate uptake kinetics by HK-2 cells. (**A**) The uptake of radiolabelled DL-Lactate (100 µM) at 10 minutes was linear. (**B**) In the presence of sodium, the uptake was pH-dependent i.e uptake was significantly (***$p<0.0001$) reduced at pH 7.4 when compared to uptake at pH 5.5. (**C**) DL-lactate uptake over a range of concentration (0.5-10 mM) resulted in apparent K_m value of 4.21± 1.0 mM (95% CI: 2.18 - 6.24) at 37 °C, pH 6.0. Data are presented as mean ± SEM of three independent experiments. Na, sodium ion.

The impact of statin on the MCT-mediated ^{3}H-labelled DL-lactate uptake

The inhibition of ^{3}H-labelled DL-lactate (100 µM) uptake by statins compared to that of phloretin, a well-known MCT1 inhibitors and CHC (a non-specific inhibitor for MCT) is shown in **figure 2.5**. Simvastatin and atorvastatin caused a similar degree of DL-lactate uptake inhibition as that for phloretin. In contrast, pravastatin, rosuvastatin and CHC caused a significantly lower ($p<0.001$) inhibitory effect on lactate uptake than that shown by phloretin; (13.22 ± 4.24 % and 51.18 ± 6.3 % for pravastatin and rosuvastatin, respectively; at a final concentration of 2 mM). Dose-response curves for DL-lactate (50 µM) uptake inhibition by statins (0.025-5 mM) are shown in **figure 2.6**. The IC_{50} values of the curves are 0.43 ± 0.2 mM, 0.60 ± 0.2 mM and are 3.06 ± 1.1 mM, for simvastatin, atorvastatin and rosuvastatin, respectively, while no IC_{50} produced with pravastatin treatment up to the highest concentration of 5 mM used.

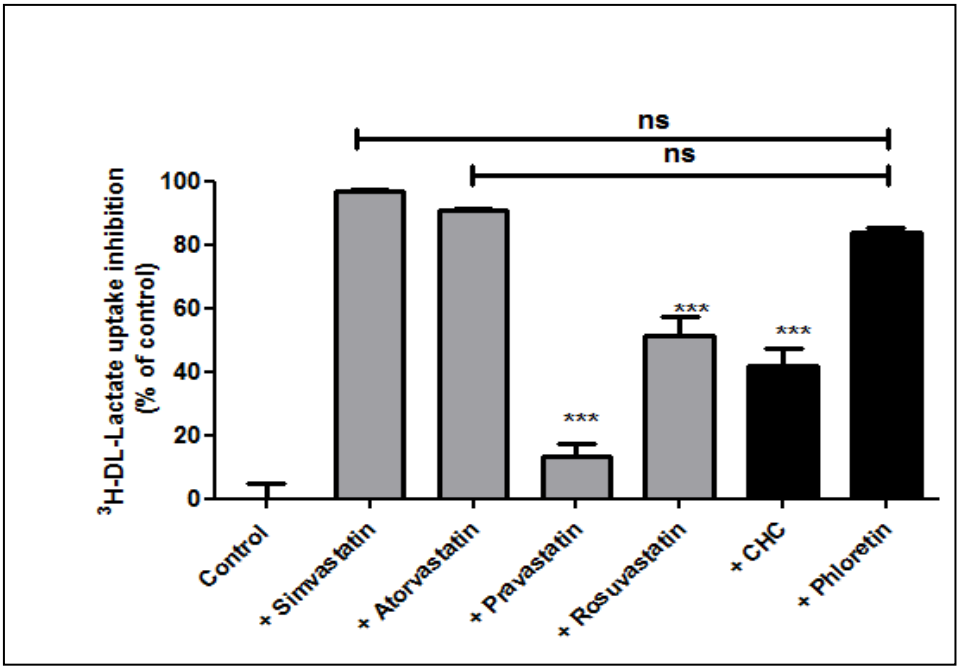
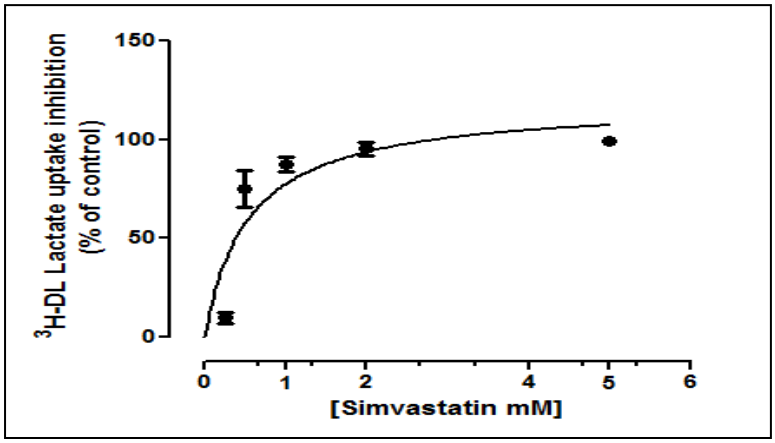


Figure 2.5 The inhibition of DL-lactate uptake (100 µM) by statins and CHC in comparison to phloretin (all at 2mM). Data are mean ±S.E.M (n = 12) from 3 independent determinations. Difference between treatment with statins and CHC and phloretin (controlled for phloretin) was tested for statistical significance by One-Way ANOVA with Dunnett's post-test. P=0.001***. CHC, α-Cyano-4-hydroxycinnamic acid *N*-ethyl-*N*,*N*-diisopropylammonium salt.

A



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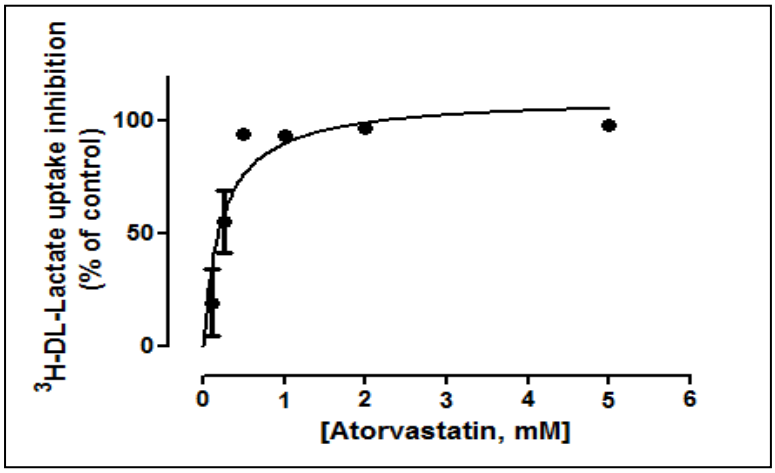

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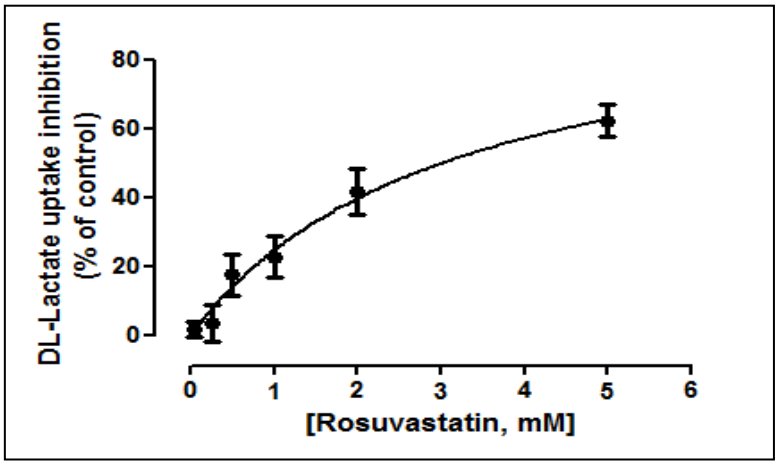


Figure 2.6 Dose-response curve for the ability of inhibitors (0.025 - 5.00 mM) to inhibit MCT-mediated DL-Lactate (50 µM) in HK-2 cells. Data are mean ± S.E.M. *n* = 12 from three independent experiments, analysed with non-linear least-squares regression. MCT, monocarboxylate transporter.

2.3.2 Expression of MCT uptake transporter in L6 rat muscle cells

Relative mRNA expression of key uptake transporters

The expression of a series of uptake transporters (both SLC and SLCO sub-groups) in the L6 cells was carried out by qPCR array (catalogue no: PARN 070Z) using total RNA isolated from L6 cells at myotube stage on day 7 after differentiation. In order to assess whether statins affect gene expression of the uptake transporters in L6 myotubes, the cells were pre-treated with simvastatin (2 µM) for 48 hrs prior to RNA extraction (day 5) and compared to untreated control cells. Among the SLC and SLCO uptake transporters, mRNA for Mct1 (Slc16a1), Mct8 (Slc16a2) and Oatp3a1 (Slco3a1) were found to be highly expressed in L6 cells in contrast to Mct4 (Slc16a3) expression (**Figure 2.7**). Moreover, the regulation of the Mct1, Mct2 and Oatp3a1 in particular, appeared not to have been much affected by simvastatin pre-treatment.

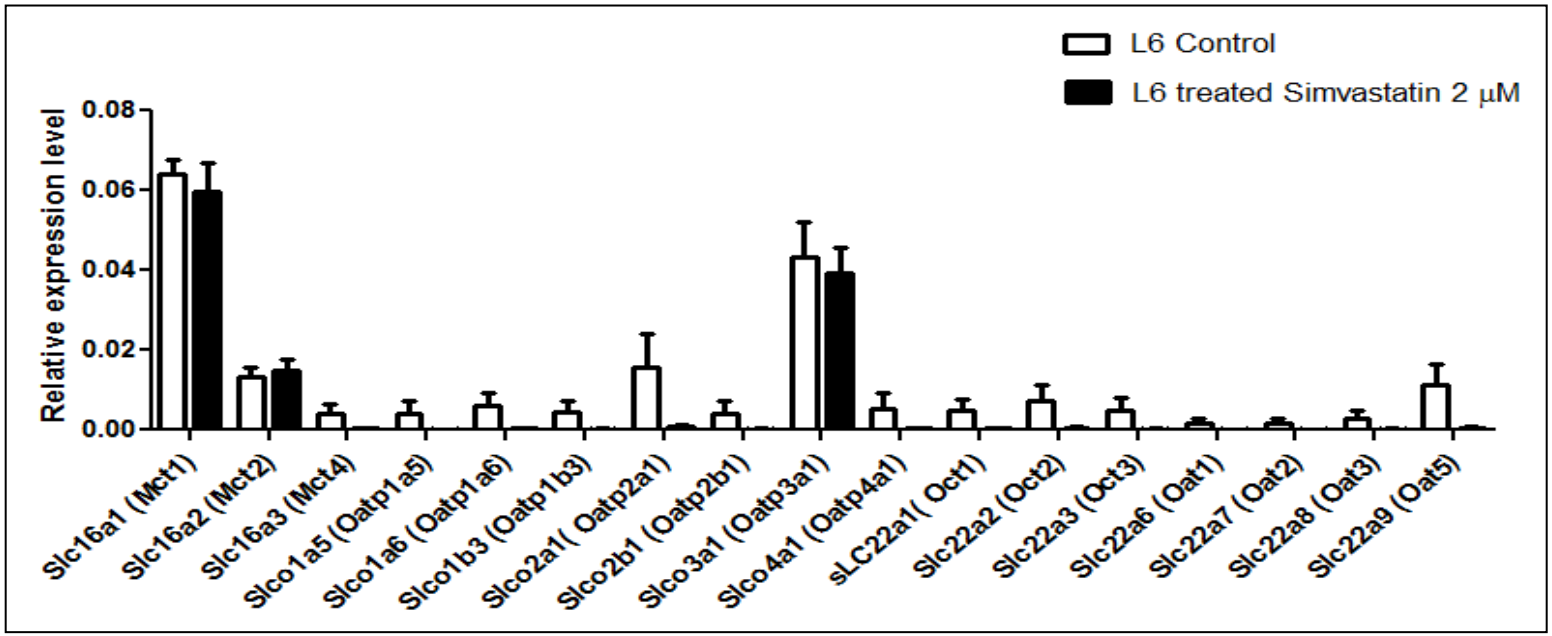
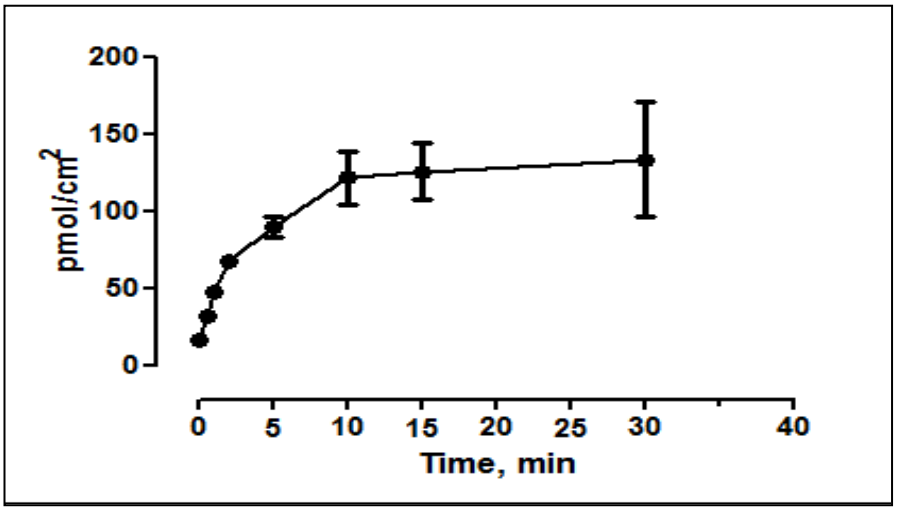


Figure 2.7 Relative transporter gene expression levels of key uptake transporters in cDNA isolated from L6 cell line at differentiated stage. The relative gene expression level was analysed by standard RT² Profiler PCR Array data analysis from SABiosciences website. The L6 cells were differentiated for 7 days and treated with simvastatin (2 µM) for 48 hrs prior to RNA extraction. The corresponding culture with vehicle solvent (MeOH, 0.02 % v/v) represents the control. The expression levels were calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to the geometric mean of 5 housekeeping genes provided by the array. Error bars represent the mean ± SEM (n=3). Mct, monocarboxylate transporter; Oatp, organic anion-transporting polypeptide; Oct, organic cation transporter; Oat, organic anion transporter; Slc, solute carrier; Slco, solute carrier organic anion family.

Optimisation of ^{3}H-labelled DL-lactate uptake as functional expression of MCT

The uptake of ^{3}H-DL-lactate (50µM) was used to assess MCT function in L6 cells. The time course for lactate uptake into L6 cells at pH 6.0 (at 37°C) is shown in **figure 2.8a**. The ^{3}H-DL-lactate uptake was linear up to 5 minutes. Therefore a 2 minutes incubation time was chosen for subsequent uptake experiments. The effect of extracellular pH and sodium ion concentration on ^{3}H-DL-lactate uptake was examined over the pH range of 5.5 to 7.4. ^{3}H-DL-lactate uptake was significantly higher ($p<0.001$) at lower extracellular pH (pH 5.5 vs pH 7.4) in the presence and absence of Na^{+}. The uptake at pH 5.5 was found not to be affected by Na^{+} concentration (**Figure 2.8b**). The uptake experiments were subsequently performed at pH 6.0 in the presence of Na^{+} to mimic physiological conditions.

A



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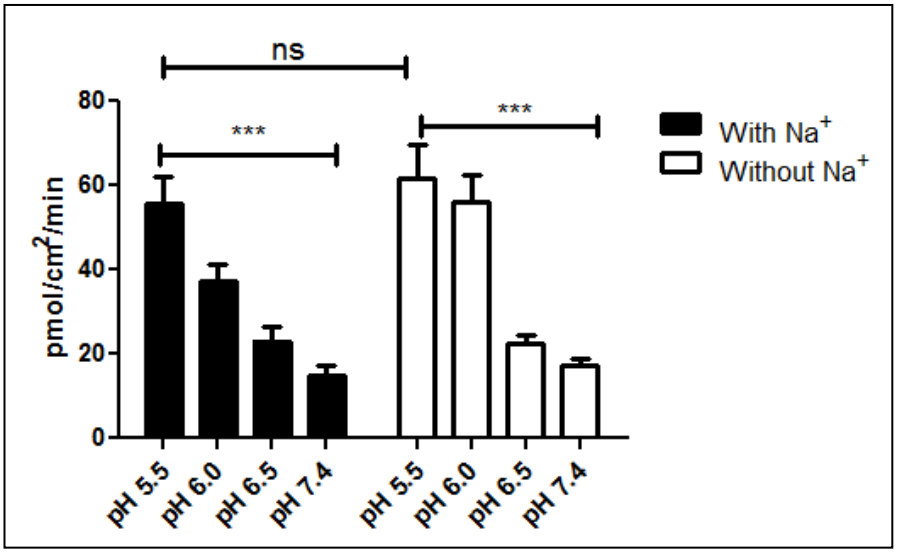


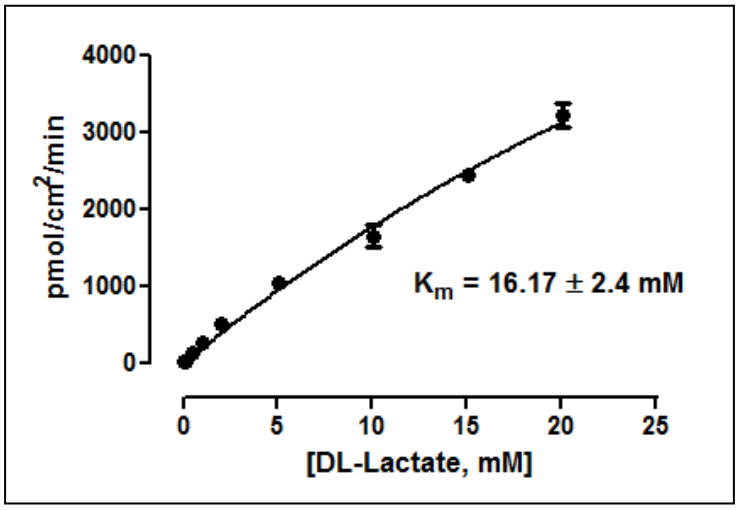
Figure 2.8 Time course and pH-dependency uptake of ^{3}H-DL-Lactate in myotubes of L6 rat muscle cells. (A) The ^{3}H-DL-Lactic acid (50 µM) uptake by L6 (at pH 6.0 and 37°C) was linear up to 5 minutes and 2 minutes incubation time was chosen for subsequent experiments. (B) Effect of pH and Na^{+} on the uptake of DL-lactate (50 µM) by L6 cells. Data are presented as mean ± SEM (n= 12) from three independent determinations. Na^{+}, sodium ion.

MCT1 function in L6 rat muscle cells

The kinetics of ^{3}H-DL-lactate uptake by the L6 cells is shown in **figure 2.9**. The uptake of DL-lactate (1 µCi/mL) was shown to be concentration-dependent (0.1 mM to 20 mM). A nonlinear regression analysis with a simple Michaelis-Menten equation gave a K_m value of 16.17 ± 2.4 mM (95% CI = 11.47- 20.87) in the presence of Na+ and the K_m value of 15.63 ± 3.0 mM (95% CI= 9.75 – 21.51) in the absence of Na+. The K_m values in both conditions were found not to be significantly different from each other (**Figure 2.10**) suggesting that in L6 cells the presence of Na^+ has no impact upon the kinetics of the transporter. This is in stark contrast to the results found with HK-2 cells.

The exact function of MCT1 transporter (i.e. either uptake or efflux of the substrate) is unclear, based upon previous reported studies in various cell lines (Bonen et al., 2000, Juel and Halestrap, 1999, Pilegaard et al., 1999, McCullagh et al., 1996), I first decided to determine MCT1 efflux capability using the ^{3}H-DL-Lactate efflux assay. The fractional efflux rate of ^{3}H-DL-Lactate (50 µM) at each time interval was low and the values were consistently at the level below than 10% from the baseline reading **(Figure 2.11**). Furthermore, the fractional efflux rates for DL-lactate were similar when co-incubated with MCT1 inhibitors, phloretin (a typical MCT1 inhibitor), CHC (non-specific MCT inhibitor), simvastatin (a representative of lipophilic statin) and pravastatin (a representative of hydrophilic statin), at a concentration of 500 µM of each inhibitor to that of control (substrate only). This finding suggests that MCT1 does not exhibit efflux function for DL-lactate in the L6 muscle cells.

A



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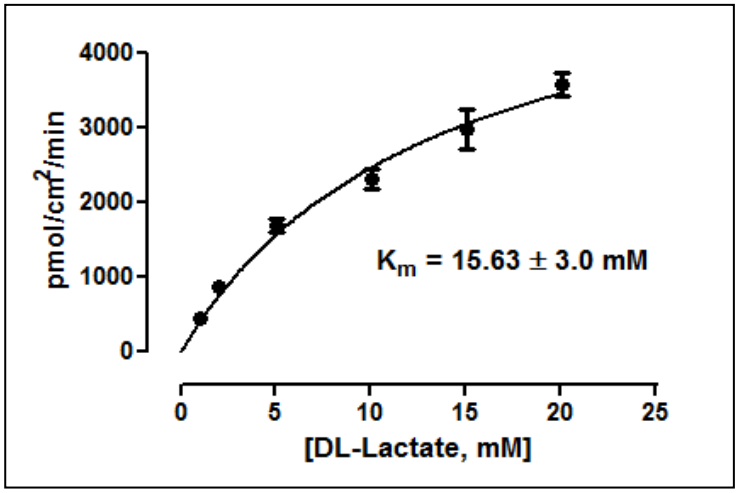


Figure 2.9 Concentration-dependence of DL-lactate uptake by L6 myotubes. ^{3}H-DL-Lactate uptake at 0.1 – 20 mM final concentration (at pH 6.0 and 37 °C) was determined over 2 minutes in (A) the presence of extracellular Na^{+} and (B) the absence of extracellular Na^{+}. The K_m values were 16.17 ± 2.4 mM (95% CI: 11.47 - 20.87) and 15.63 ± 3.0 mM (95% CI: 9.75 – 21.51) in the presence and absence of Na^{+}, respectively. Data are presented as mean ± SEM from three independent determinations. Na^{+}, sodium ion.

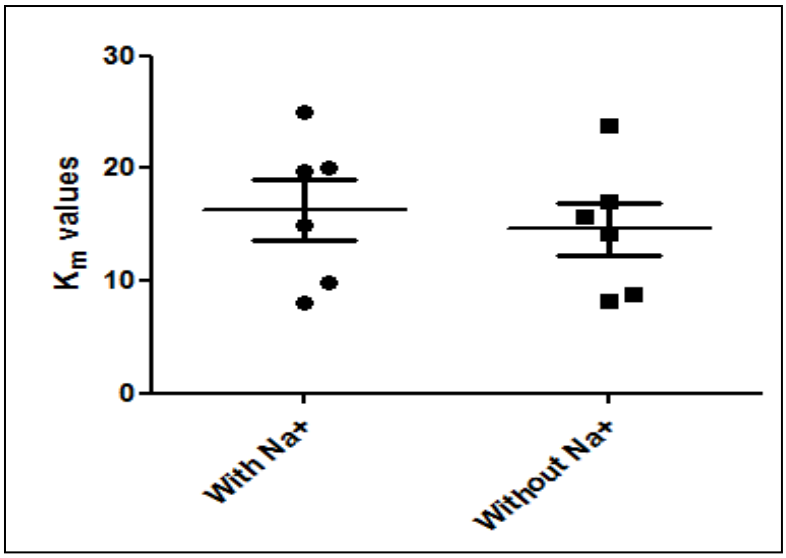


Figure 2.10 Comparison of K_m values in the presence and absence of extracellular Na^+. The mean K_m value in the presence of Na^+ (16.17 ± 2.4 mM) was not significantly different (paired t-test) compared to that without Na^+ (15.63 ± 3.0 mM). Data are presented as mean ± SEM (n = 6) from six parallel determinations. Na^+, sodium ion.

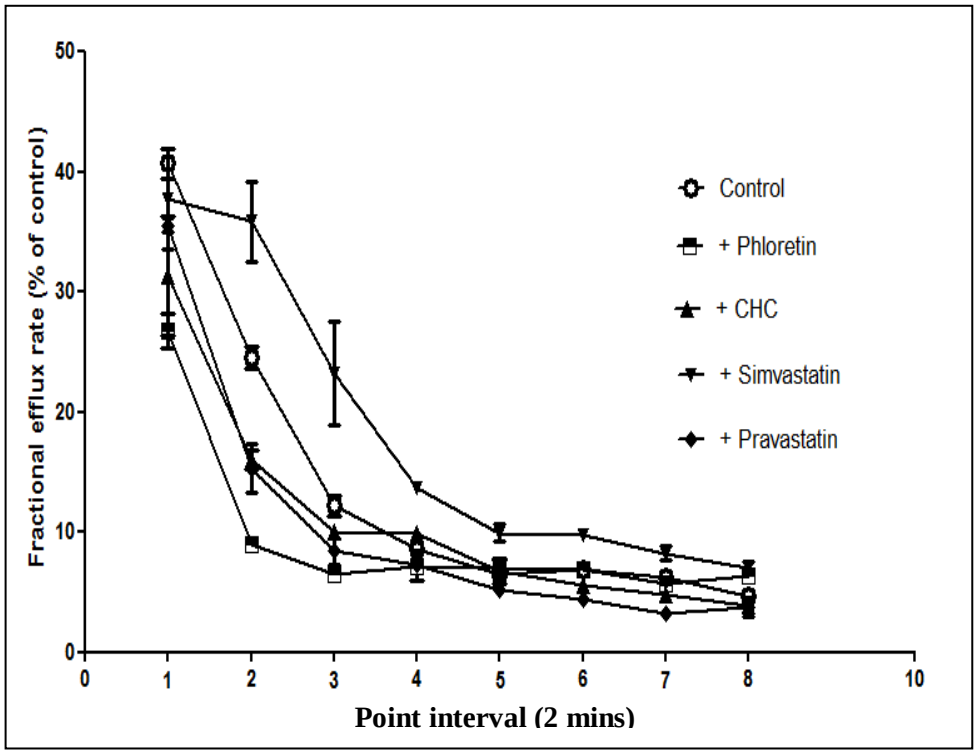


Figure 2.11 Efflux assay for determination of MCT1-mediated ^{3}H-DL-Lactate (50 µM) efflux in L6 cells. There was no significant inhibition of DL-lactate (50 µM) efflux. Efflux rates were approximately 10 % for all conditions. All inhibitors were at 500 µM and each point represented as mean ±SEM (n =9) from three independent experiments. MCT, monocarboxylate transporter; CHC, α-Cyano-4 hydroxycinnamic acid *N*-ethyl-*N*,*N*-diisopropylammonium salt.

The impact of statin on the MCT-mediated ^{3}H-labelled DL-lactate uptake

Inhibition assay was performed to determine whether statins affect functional expression of Mct1 in particular. L6 cells were incubated with ^{3}H-DL-lactate in the absence and the presence of statins (i.e. simvastatin, atorvastatin, pravastatin and rosuvastatin). To confirm that statin was a substrate for Mct1, the magnitude inhibition of DL-lactate uptake by all tested statins were compared to phloretin (a well-defined Mct1 inhibitor) and CHC (a typical Mct1, 2 and 4 inhibitor). **Figure 2.12** summarises the degree of DL-lactate uptake inhibition. Simvastatin and atorvastatin significantly ($p<0.001$) inhibited DL-lactate uptake to the same degree as phloretin and CHC with IC_{50} values of 10.7 ± 1.2 µM and 7.4 ± 0.9 µM, respectively (**Table 2.1**). In contrast, the inhibitory effects of pravastatin and rosuvastatin were weak even up to 1 mM (**Figure 2.12**).

The IC_{50} (i.e., below 100 µM) that resulted in 50% of maximal DL-lactate uptake were independently determined **(Appendix D**); they did not affect L6 cells' viability for up to 48 hours post-treatment.

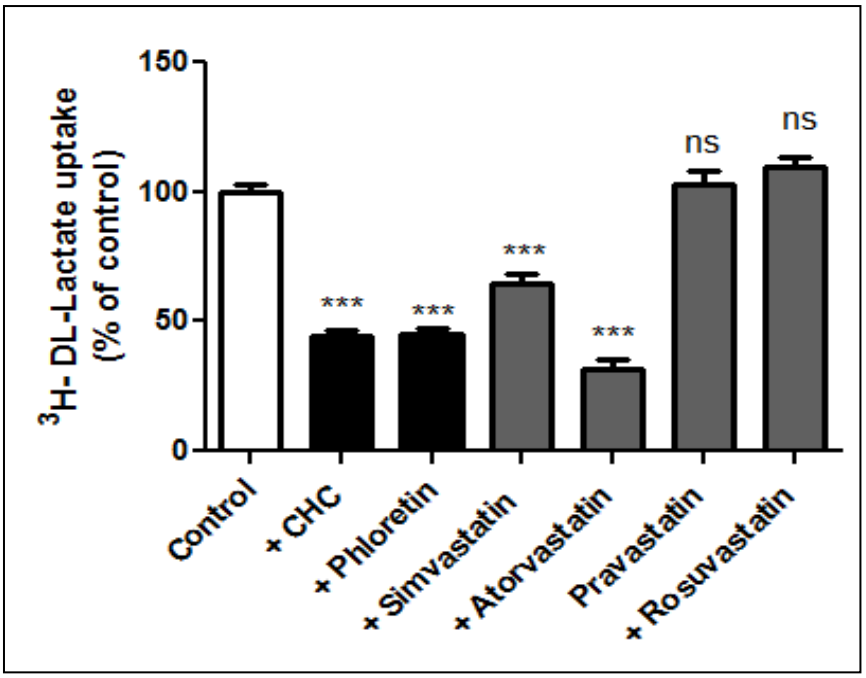


Figure 2.12 **The DL-lactate uptake (50 µM) in the presence of statins, CHC and phloretin (all at 1 mM).** Data are mean ±S.E.M. n = 12, each data point was derived from a triplicate of experiments and from four independent determinations. Data were analysed using One-Way ANOVA with Dunnett's post-test and compared to that of control without the presence of inhibitor. (*** $p < 0.001$); ns, non-significant. CHC, α-Cyano-4 hydroxycinnamic acid *N*-ethyl-*N*,*N*-diisopropylammonium salt.

Table 2.1 IC_{50} and V_{max} values for the ability of the agents to inhibit ^{3}H-DL-Lactate (50 µM) uptake into L6 myotubes.

Inhibitors	IC_{50} values (95% CI)	V_{max} (mean ± SEM)
Phloretin	8.8 ± 0.7 µM (7.43 – 10.17)	72.07 ± 0.9 %
Simvastatin	10.7 ± 1.2 µM (8.35 – 13.05)	61.30 ± 1.1 %
Atorvastatin	7.4 ± 0.9 µM (5.64 – 9.16)	69.60 ± 0.8 %

2.3.3 Expression of MCT transporters in cultured human muscle cells

Relative mRNA expression of key uptake transporters

The expression of a series of uptake transporters (both SLC and SLCO sub-groups) in human muscle cells was carried out by qPCR array (catalogue no: PAHS 070Z). It was performed using total RNA isolated from myotubes of human muscle cell line on day 7 after differentiation. Among uptake transporters, only mRNA for MCT1 (SLC16A1) was detected, though OATP3A1 and MCT8 (SLC16A2) were also detected but at very low levels.

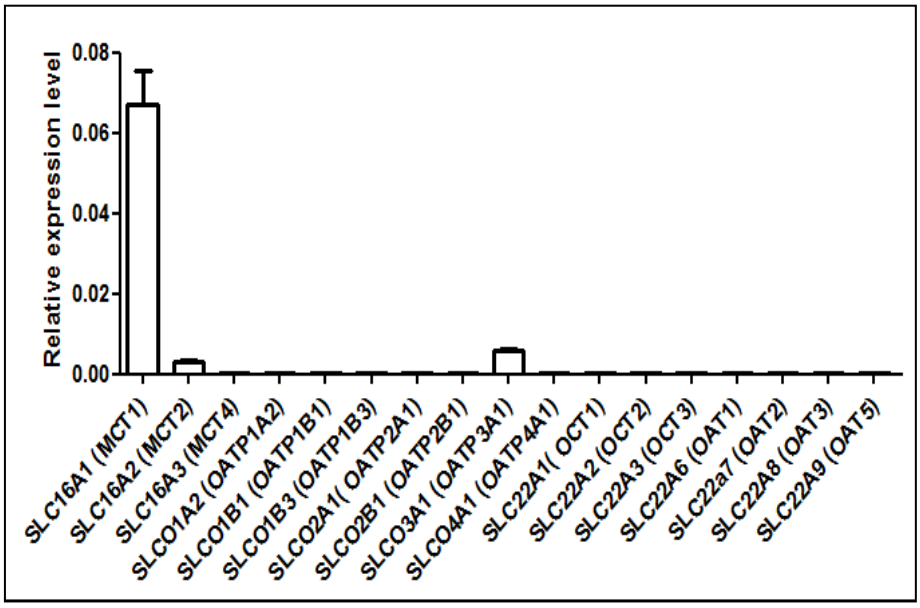


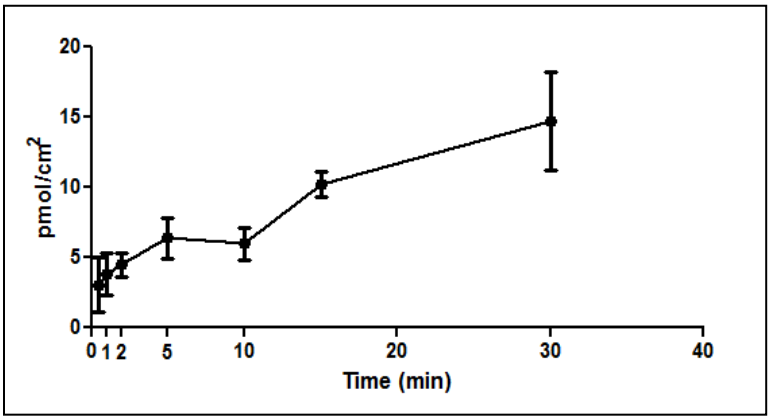
Figure 2.13 Relative transporter gene expression levels of key uptake transporters in cDNA isolated from human muscle cells at myotube stage. The relative gene expression level was analysed by standard RT² Profiler PCR Array data analysis from SABiosciences website. The expression levels were calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to the geometric mean of five housekeeping genes provided by the array. Error bars represent the mean ± SD of two independent assays of two separate cultures of human muscle cells. MCT, monocarboxylate transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; OAT, organic anion transporter; SLC, solute carrier; SLCO, solute carrier organic anion family.

Optimisation of ^{3}H-labelled DL-lactate uptake as functional expression of MCT

Tritium radiolabelled DL-lactate was used to assess MCT function in human muscle cells. The time course of ^{3}H-DL-lactate (50 µM) uptake into human muscle cells and the impact of pH on uptake are shown in **figure 2.14a**. Similar to the L6 cells, the ^{3}H-DL-lactate uptake was linear up to 5 minutes and thus 2 minutes incubation time was chosen for subsequent uptake experiments. However, DL-Lactate uptake at the abovementioned concentration in myotubes of human muscle cells was significantly lower ($p<0.0001$) compared to that seen in L6 cells (6.45 ± 1.2 pmol/cm^2/min vs 37.17 ± 4.3 pmol/cm^2/min at 2 mins) under the same conditions (pH 6 and 37°C, in the presence of Na^+).

The effect of extracellular pH and sodium ion concentration on ^{3}H-DL-lactate uptake was then examined over the pH range of 5.5 to 7.4. As with L6 myotubes (**section 2.4.2**), the uptake of the ^{3}H-DL-lactate in human myotubes significantly increased ($p<0.05$) with increasing acidic extracellular pH (pH 5.5 vs pH 7.4) in the presence of Na^+. While at the lower extracellular pH of 5.5, the DL-Lactate uptake was found to be not affected by Na^+ (**Figure 2.14b**).

A



B

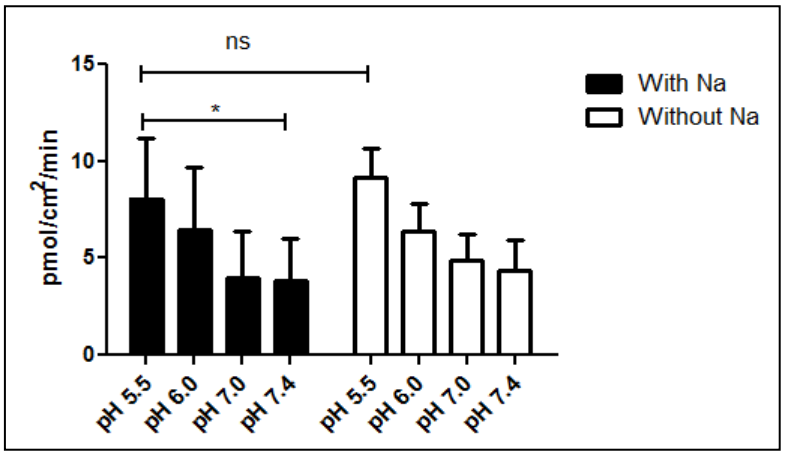


Figure 2.14 Time course and pH-dependency of ^{3}H-DL-Lactate uptake in myotubes of human muscle cells. (**A**) The ^{3}H-DL-Lactic acid (50 µM) uptake by human muscle cells (at pH 6.0 and 37°C) was linear up to 5 minutes and 2 minutes incubation time was chosen for subsequent experiments; (**B**) Effect of pH and Na^{+} on the uptake of DL-lactate (50 µM) by human muscle cells. Data are presented as mean ± SEM (n= 12) from three independent determinations. Na^{+}, sodium ion.

Functional expression of MCT uptake transporter in human muscle cells

The kinetics of ^{3}H-DL-lactate uptake by human muscle cells is shown in **figure 2.15**. Similar to the L6 myotubes (**section 2.3.2)**, the uptake of ^{3}H-DL-lactate (1 µCi/mL) was shown to be concentration-dependent (0.1 mM to 20 mM). A nonlinear regression analysis with a simple Michaelis-Menten equation gave a K_m value of 9.66 ± 2.4 (95% CI: 4.96 – 14.36) mM in the presence of Na^+ and the K_m value of 9.85 ± 4.7 (95% CI: 0.37-19.32) mM in the absence of Na^+. However, the uptake value presented at the concentration of 20 mM in human muscle myotubes were not significantly different to that shown by L6 myotubes.

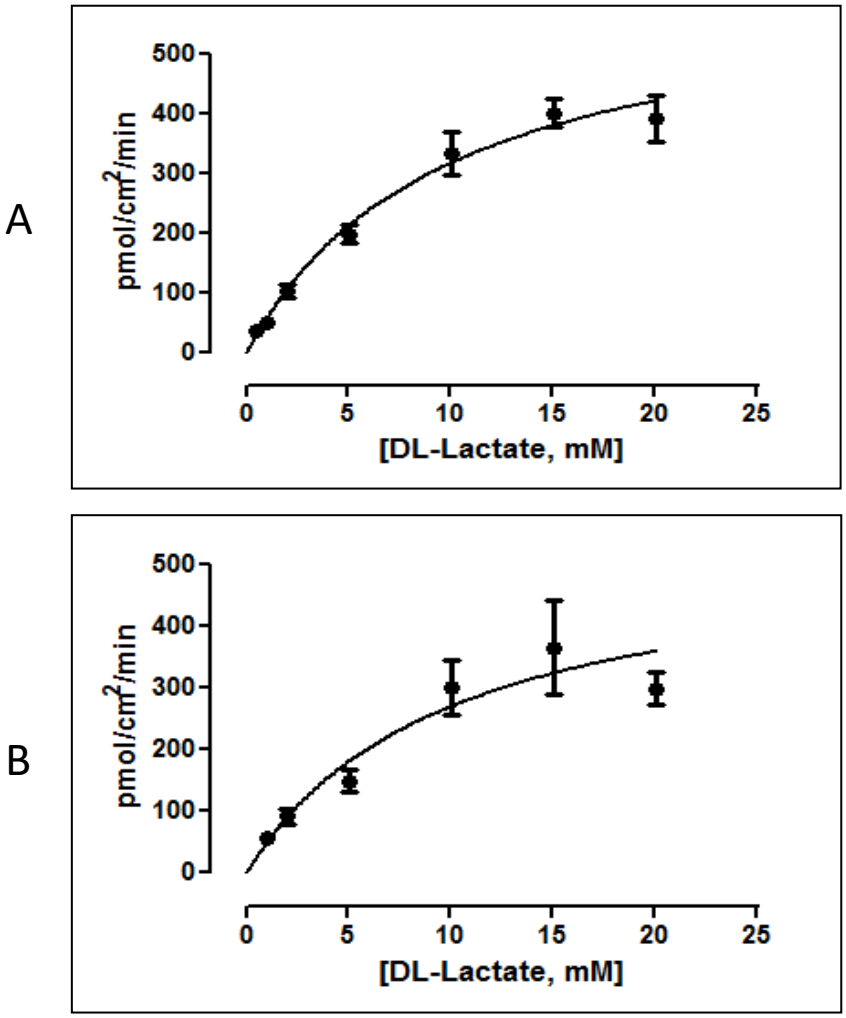


Figure 2.15 Concentration-dependence of DL-lactate uptake by human muscle myotubes. ^{3}H-DL-Lactate uptake; 0.1 – 20 mM (at pH 6.0 and 37°C) was determined over 2 minutes in (A) the presence of extracellular Na^+ and (B) Na^+-free conditions. The K_m values resulted from the DL-Lactate uptake is 9.66 ± 2.4 (95% CI: 4.96 – 14.36) mM and 9.85 ± 4.7 (95% CI: 0.64 -19.03) mM in the presence and absence of Na^+, respectively. Data are presented as mean ± SEM from three independent determinations. Na^+, sodium ion.

The impact of statin on the MCT-mediated ^{3}H-labelled DL-lactate uptake

Inhibition assay was performed to determine whether statins affect functional expression of MCT1 in particular. Human muscle cells were incubated with ^{3}H-DL-lactate in the absence and the presence of statins (i.e. simvastatin, atorvastatin, pravastatin and rosuvastatin) and phloretin. To confirm that statin was a substrate to MCT1, the inhibition of DL-lactate uptake by all tested statins were compared to phloretin (a well-defined MCT1 inhibitor). **Figure 2.16** summarises the relative uptake of DL-lactate (50 µM) with and without the presence of the agents. At concentration of 500 µM, simvastatin and atorvastatin significantly ($p<0.001$) inhibited DL-lactate (50 µM) uptake to the same degree as phloretin. In contrast, pravastatin and rosuvastatin were weak inhibitors of lactate uptake.

Figure 2.17 and **Table 2.2** show the IC_{50} and V_{max} values of simvastatin and atorvastatin compared to those of phloretin. The IC_{50} value for simvastatin (211.8 ± 65.37 µM) was five-fold higher than that for both phloretin (45.58 ± 17.15 µM) and atorvastatin (42.82 ± 15.06 µM). However, the V_{max} for both simvastatin and atorvastatin were similar to that for phloretin.

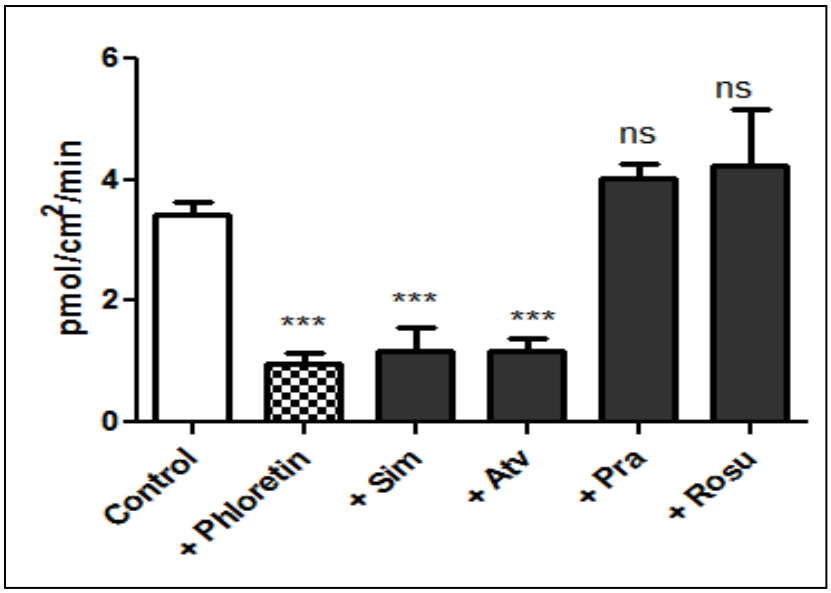


Figure 2.16 The inhibition of DL-lactate uptake (50 µM) by statins and phloretin; all at 500 µM final concentration. Data are mean ±S.E.M (n = 9) from 3 independent determinations. Difference between treatment with statins and phloretin (controlled for wells without inhibitor) was tested for statistical significance by One-Way ANOVA with Dunnett's post-test. *** p= 0.001.Sim, simvastatin; Atv, atorvastatin; Pra, pravastatin; Rosu, rosuvastatin.

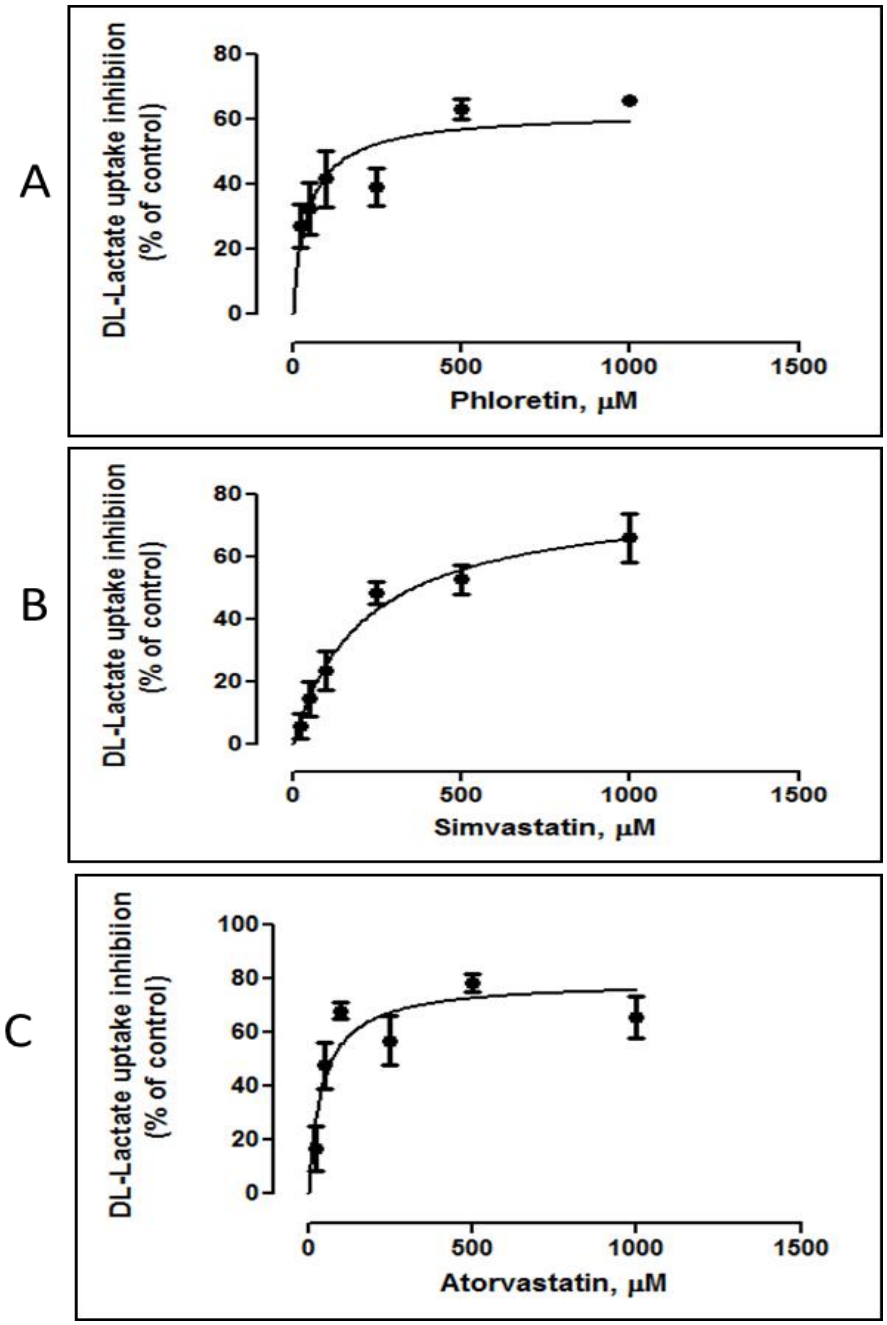


Figure 2.17 Dose-response curve for DL-lactate uptake in the presence of phloretin. (A), simvastatin (B) and atorvastatin (C) in cultured human muscle cells. Data are presented as mean ± S.E.M. n = 12 from three independent experiments, analysed with non-linear least-squares regression.

Table 2.2 IC_{50} and V_{max} values for phloretin, simvastatin and atorvastatin to inhibit ^{3}H-DL-Lactate (50 µM) in human muscle myotubes.

Inhibitors	IC_{50} values, mean ± SEM	V_{max} (mean ± SEM)
Phloretin	45.58 ± 17.15 µM	62.01 ± 5.76 %
Simvastatin	211.8 ± 65.37 µM	79.64 ± 9.31 %
Atorvastatin	42.82 ± 15.06 µM	78.97 ± 7.07 %

2.4 Discussion

The aim of this study was to investigate the effect of statins on transmembrane transporters which might be associated with their myotoxicity. The study mainly focused on the impact of statins on monocarboxylate transporters (MCTs), since they are abundantly localised in skeletal muscle, and to evaluate the possible effect of mutations on the functional activity of the transporters with a view to their selection as part of a later case-control study evaluating the impact of environmental and genetic factors on the susceptibility to statin-related myotoxicity (Chapter 4).

The impact of statins on the functional expression of MCTs was first examined in an *in vitro* cell-based system using MCT-expressing cell line, HK-2 (originated from proximal tubule cell in human kidney), before embarking the same-designed functional studies in a model of muscle originated from both rats and humans. We have, previously, shown both mRNA and functional expression of MCT-1 in HK-2 cells, using DL-lactate as substrate (Jenkinson et al., 2012). DL-lactate uptake in HK-2 cells was found to be transported by mechanism obeying Michaelis-Menten kinetics (K_m), indicating transporter-mediated uptake, likely through the MCT1 transporter which was the major SLC16A transporter found in the cells although the Na^+ dependence of the uptake suggested that the uptake may have been mediated by SMCT1 (sodium-coupled MCT1) rather than MCT1. The K_m value (95% CI: 2.25 - 6.17) for the DL-lactate uptake in the HK-2 cells was lower compared to that seen in L6 rat muscle (95% CI: 11.47 - 20.87mM) and human muscle cells (95% CI: 4.96 – 14.36 mM). The K_m value in the HK-2 cells was, however, about the same magnitude to that found with L-lactate (3.5 ± 0.4 mM) transfected with MCT1 cRNA in heterologous system *Xenopus laevis* oocytes expression system (Broer et al., 1998). It might be possible that lactate kinetics were dependent on the nature of cell culture-based system in which tissue it has been derived from, thus suggesting the kinetic could be different among different tissues. Relatively, the mRNA expression level for MCT1 in the HK-2 cells was higher than L6 rat and human muscle cells (1.0 vs 0.06, in HK-2 cells and both L6 and human muscle cells, respectively), suggesting its lower affinity to DL-Lactate in both muscle models. Likewise, it could be possible that HK-2 cells extensively transport or eliminate lactate as metabolite substrate and/or product of the endogenous production, thus resulting in lower K_m values (high driving forces) than

that of muscle cells, since HK-2 cells were found to express more MCTs; MCT1, MCT2 and MCT4, at both mRNA and protein levels (Wang et al., 2006).

In the present study, myotubes originated from human and rat muscle cells were used as models for muscle to determine species differences, if any, in the kinetic of lactate uptake. Human and L6 rat muscle were found to have a K_m value within the range of values obtained in the other studies on lactate transport in skeletal muscles. The K_m values from both models match those observed in earlier studies. Previous reports from tracer studies have shown that K_m values range from 13 mM in intact skeletal muscle to 40 mM in vesicles isolated from rat skeletal muscle (McDermott and Bonen, 1993a, McDermott and Bonen, 1993b, Juel, 1991, Roth and Brooks, 1990) to 12.5 mM in myotubes of L6 cell line (El Abida et al., 1992). Similarly, the K_m for lactate transport prepared from human skeletal muscle is within the same range as the values obtained in sarcolemmal giant vesicles from rats (Juel et al., 1994). It can thus be suggested that the L6 rat muscle cells could be used as a model for MCT1 function that may resemble its functional expression in cell line as well as intact skeletal muscle originated from human.

In contrast to the findings in HK-2 cells (Jenkinson et al., 2012), I found that the DL-lactate uptake in L6 cells was Na^+-independent. Since the absence of SMCT1 in the HK-2 cells was verified by RT-PCR, it was suggested that the inhibition of Na^+/H^+ exchange in the HK-2 cells resulted in higher lactate uptake in the presence of Na^+ (Jenkinson et al., 2012). Therefore, one possible explanation of this discrepancy could be the result of high regulation of lactate/H^+ exchange since it was found as major pH regulator in muscle cell compared to other mechanisms such as Na^+/H^+ exchange and bicarbonate/H^+ (Juel, 1995). It seems that only the presence of excess proton (H^+) intensifies the lactate transport, in accordance with other previous findings (Halestrap and Price, 1999, Broer et al., 1998) and thus suggests that muscle symptoms among lipophilic statin users in particular, is associated with the disturbance of pH regulation in muscle and eventually lactic acidosis which might lead to apoptosis and toxicity.

To date 18 isoforms of MCTs have been characterised with MCT1 and MCT4 being the only major isoforms found in skeletal muscle (Bonen et al., 2000, Halestrap and Price,

1999). Therefore, the investigation as to whether statins functionally affect MCT transporter isoforms (either MCT1 or MCT4) might provide insight into the mechanism underlying SRM. It is interesting to note that lipophilicity of statins determine the affinity to the MCT1 suggesting that the SRM symptoms, among simvastatin and atorvastatin users particularly, might involve muscular lactate metabolism damage as a result of reduced lactate uptake and/or efflux inhibition. Since phloretin did not significantly inhibit lactate efflux in the L6 cells, then it can be inferred that Mct1 transporter in this cell line did not exhibit efflux function which is consistent with previous finding by Kobayashi and colleagues (Kobayashi et al., 2004). In addition, others have shown that MCT1 is specifically involved with lactate uptake (Juel and Halestrap, 1999, Pilegaard et al., 1999, McCullagh et al., 1996) while lactate efflux is mediated by MCT4 (Bonen, 2001, Wilson et al., 1998). Although it could be worth determining as to whether statins specifically inhibit lactate efflux by MCT4, (unfortunately, we did not undertake any specific functional studies of MCT4 to implicate any meaningful MCT-specific analysis) which might be the reason for SRM as a result of depressed muscle function due to lactic acidosis, as explained by new paradigm for lactate metabolism (Gladden, 2004). This also corroborates with earlier findings (Kobayashi et al., 2006, Sirvent et al., 2005) that statins also affect MCT4 transporter function and could be of interest for future research, especially using specific transfection or site-directed mutagenesis of the gene.

The cellular studies in this project have several limitations. Some statin uptake transporters, which were shown in previous studies, have not been expressed in at the messager level. Among the three cell lines studied, there was no expression of OATP1B1 transcribing gene (SLCO1B1), the only transporter showing significant association with statin-induced muscle toxicity, and are thus poor models for functional studies of the OATP1B1 transporter. In this study, expression at the messager level for transporter such as OATP2B1 (Knauer et al., 2010), human organic anion transporter (OAT) 1 and OAT3 (Takeda et al., 2004), were not exhibited in the human myotubes. It could be due to reduced expression events that may happen during substantial differentiation as cell lines with high passage numbers are more likely to exhibit alterations in cell morphology and functions. The skeletal muscle myoblast cells, however, was found to exhibit multinucleated characteristic of myotube feature up to 8 passage number under light microscopy observation. For future experiments, it is suggested to perform experiments using newly established low-passage primary myoblast cultures up to 3 or 4 passage

number only. There was also some limitations in term of the protocols used for functional assays described in the experiments. Relative uptake of the radiolabeled substrate into cell monolayers was expressed in terms of cell monolayer surface area (exhibited as cm^2 in the **equation 2.1**) rather than the usual protocol of using cells number or protein concentration. Estimation of the substrate uptake according to the cell monolayer surface is subjective, and therefore is dependent on the extent of cell confluence. This deficiency might explain large SEM for the uptake values between replicates for certain result of experiments.

In the present study, it was interesting to note that the affinity of both simvastatin and atorvastatin to the lactate uptake inhibition were different between cells originated from kidney and muscle (i.e., lower IC_{50} values for both statins in muscle cells). It seems that the affinity of statins to MCTs was likely dependent on their lipophilicity. The more lipophilic statins were expected to have higher affinity to MCTs and their affinity as substrates and/or inhibitors to the MCTs was higher to muscle cells than cells originated from kidney cells, the HK-2 cells. However, the reason as to why the affinity of the lipophilic statins was higher in muscle models is unclear. It is possible that co-expression of MCT4 function, though not detectable at the mRNA, in the HK-2 cells interfers with their affinity to the inhibition of MCT-mediated DL-lactate uptake. This is supported by the observation of the degree of DL-lactate uptake inhibition by CHC, a competitive inhibitor for MCT1 and MCT4 (Manning Fox et al., 2000) in HK-2 and L6 muscle cells. The capacity of CHC to inhibit the DL-lactate uptake in the HK-2 cells was not in the same magnitude as phloretin's, which might be consistent with the existence of MCT4 in HK-2 cells, but not in the L6 cells. CHC did not completely inhibit the DL-lactate uptake in the HK-2 cells probably due to its binding to MCT4 as well. The difference in the sensitivity of CHC between MCT1 and MCT4, however, is unclear at the present time, and so further detailed investigations are needed to clarify this.

The affinity of atorvastatin for MCT1 in the cells was five-fold higher than that of simvastatin. It seems that atorvastatin, although at lower concentration than that of simvastatin, would have resulted in higher inhibition to MCT1-mediated lactate transport, thus suggesting atorvastatin-related muscle symptoms are related to the MCT1 inhibition. This is explained by its lack-of- dose-dependency manner (10 mg versus 80mg) to induce

muscle adverse events (Athyros et al., 2010, Newman et al., 2006, LaRosa et al., 2005). Compared with simvastatin, atorvastatin was proven to be more safe, when evaluated in more than 11,000 patients, in which at higher dose, the rate of clinically significant atorvastatin-induced myopathy was very low (Waters, 2005). In contrast, simvastatin is more likely to induce SRM at higher dose (80 mg) (Armitage, 2007, Thompson et al., 2006). In skeletal muscle, lower affinity of simvastatin to MCT1 results in higher competition with other MCT1's substrates, and thus only simvastatin at higher dose will induce the same degree of muscle toxicity. Atorvastatin, regardless of the dose, will compete with other MCT1's substrate and/or inhibit lactate transport and eventually lead to muscle toxicity as a result of lactate transport defect. This phenomenon is more likely to be more obvious in high lactate level condition such as during exercise or the presence of DDI with other substrates of MCT1. In a blinded, controlled trial (The Effect of Statins on Skeletal Muscle Function and Performance, STOMP) for example, the detrimental muscle effects (with increased CK levels) was confirmed for individuals with physical activity or exercise while treated with atorvastatin (Parker et al., 2013) indicating that atorvastatin-induced muscle symptoms were exacerbated by high lactate levels.

In terms of localization, MCT1 in particular, has been found to be expressed abundantly in muscle and in mitochondria sarcolemmal (Butz et al., 2004, Brooks et al., 1999). Current literature has highlighted a possible association between SRM and structural and functional mitochondria damage (Larsen et al., 2013, Stringer et al., 2013, Golomb and Evans, 2008, Sirvent et al., 2008), suggesting that MCT1 inhibition might result in muscle symptoms in relation to damage caused by lactate oxidation at the cellular and/or mitochondrial level. Several case reports have also reported statin myopathy exacerbated by exercise (Parker and Thompson, 2012, Thompson et al., 1997) which might be due to interference with lactate transport by circulating statins. In term of muscle mass, older individuals (El-Salem et al., 2011, Szadkowska et al., 2010) and females (although no strong evidence found for this group) tend to have lower muscle mass compared to their counterparts (Schech et al., 2007). This group of patients may therefore be more vulnerable to SRM possibly due to relatively lower MCT expression, consistent with lower muscle mass, to accommodate with high statin plasma level. These possibilities warrant further studies.

In conclusion, the lipophilicity of a statin influences its affinity to MCT1 transporter in kidney and muscle cells, and MCT1 may be a factor in mediating statin muscle tissue exposure and toxicity.

Chapter 3. Effects of Statins on Functional Expression of Efflux Transporters

3.1 Introduction

As highlighted in the introduction (**Section 1.3**) and the findings in Chapter 2, research into the genetic influences of ABC transporters in relation to statin toxicity has gained attention among researchers (Hillgren et al., 2013).

I sought to investigate the possible impact of statins on the functional expression of ABC transporters in muscle cells. Previously our group had established that 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid (MK571) could be used as a high affinity MRP inhibitor in an MRP-mediated CMFDA efflux assay using HK-2 cells (Jenkinson et al., 2012). A dual dye assay was developed for the evaluation of the impact of two inhibitors, MK571 (MRP inhibitor) and CSA (MDR1 inhibitor); the accumulation of dye providing an indirect measure of efflux inhibition of the ABC transporters. Therefore, using the same approach, functional studies of efflux transporters were carried out in both animal muscle (L6) cells and human muscle cells. MDR1/mdr1a activity was assessed indirectly by measuring the inhibition of Hoechst 33342 (H33342) dye efflux from the cell by cyclosporine A (CSA). The ability of MK571 to inhibit CMFD dye efflux was used to assess activity of the multidrug resistance proteins (MRP/mrp). In order to assess the relative affinity of different statins to MDR1 and MRPs, the magnitude of the dye efflux inhibition was compared to that by their typical inhibitor, CSA and MK571, respectively.

3.2 Materials and Reagents

RT^2 Profiler^{TM} Human Drug Transporter PCR array (Catalogue no: PAHS-070Z,) and Rat Drug Transporter PCR array (PARN-070Z), both from Qiagen Ltd (Crawley, UK) as indicated in **section 2.2**, was used to determine relative gene expression level for efflux transporters. Reagents for dye assay; 5-chloromethylfluorescein diacetate (CMFDA) was purchased from Invitrogen (Paisley, UK), Cyclosporine A (CSA) from CalBioChem (UK). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (Dorset, UK) and were of the highest quality available.

3.3 Methods

3.3.1 Dye efflux assay

The general principle of this assay relied on the ability of the cells to retain fluorescent substrate in the presence of inhibitors, which is dependent on the functions of the substrate transporters (**Figure 3.1**). In this case, functional activities of MDR1 and BCRP were determined by the level of intracellular retention of substrate H33342, and the activity of MRPs by the retention of CMFDA product fluorescent, GSMF (glutathione methylfluorescein). Specific assays are described in the following sections.

3.3.2 Assay principle: MDR1-mediated Hoechst 33342 efflux assay

The H33342 dye can be used to measure the functional expression of MDR1 and BCRP. This dye diffuses easily across cell membranes. Upon binding with DNA, H33342 becomes fluorescent with excitation spectral maxima at 367 nm and broad emission spectra with maxima 495 nm (Kasten, 1999). In the absence of efflux transporters MDR1 and BCRP, for both of which H33342 is a substrate, it establishes equilibrium between inter- and extracellular environments. If MDR1 or BCRP are present, H33342 is actively effluxed from the cell causing a drop in intracellular fluorescence levels. This efflux can be inhibited by the introduction of any agent that interferes with the efflux activity of the transporters, resulting in an increase in intracellular fluorescence (Muller et al., 2007).

3.3.3 Assay principle: MRP-mediated CMFDA efflux assay

CMFDA, 5-chloromethylfluorescein-diacetate, is a non-fluorescent, lipophilic derivative of fluorescein, which easily permeates across the cell membrane. Inside the cells it is cleaved by unspecific esterases forming the fluorescent intermediate 5-chloromethylfluorescein (CMF). CMF is hydrophilic and exhibits a very slow permeation across cell membranes. In a second step, the chloromethyl group reacts with intracellular thiol groups such as glutathione to produce GSMF (**Figure 3.1**), which is much more hydrophilic. GSMF is a substrate of MRPs and is actively excreted out of the cells. Interactions of test-compounds with MRPs result in an increase in intracellular fluorescence intensity (Förster et al., 2008). FLUOstar Omega plate reader detects the fluorescence intensity for CMFDA at the excitation wavelength of 480 nm ± 5 nm and

emission of 520 nm ± 5 nm. The gain was set at the beginning of each experiment for the plate where highest fluorescence was expected and then kept constant throughout the experiment. The gain was consistently set to 4500 ± 250 units throughout all experiments. All experimental solutions were read on the instrument compared to water background and did not quench the fluorescence signal for the dye.

3.3.4 Dual dye retention assay

According to the knowledge of transporter-mediated substrate dye efflux, dual dye retention assay could be performed to functionally characterise MRPs and MDR1 transporter simultaneously. We previously confirmed the dual dye retention assay as a novel approach to functionally characterise both transporters simultaneously (Jenkinson et al., 2012), and it was further confirmed in the present study in the HK-2 cells and the L6 cells. Non-overlapping in signal was detected for GSMF at emission wavelength of 480 nm (within spectral range to detect free H33342 / H33342-DNA) and to an emission wavelength of 520 nm (within spectral range to detect GSMF). This will be an effective strategy to allow high throughput screening for functional expression of efflux transporters, one in which MRP and MDR1 transporters were evaluated in parallel using particular fluorescent probe specific to the transporter, respectively.

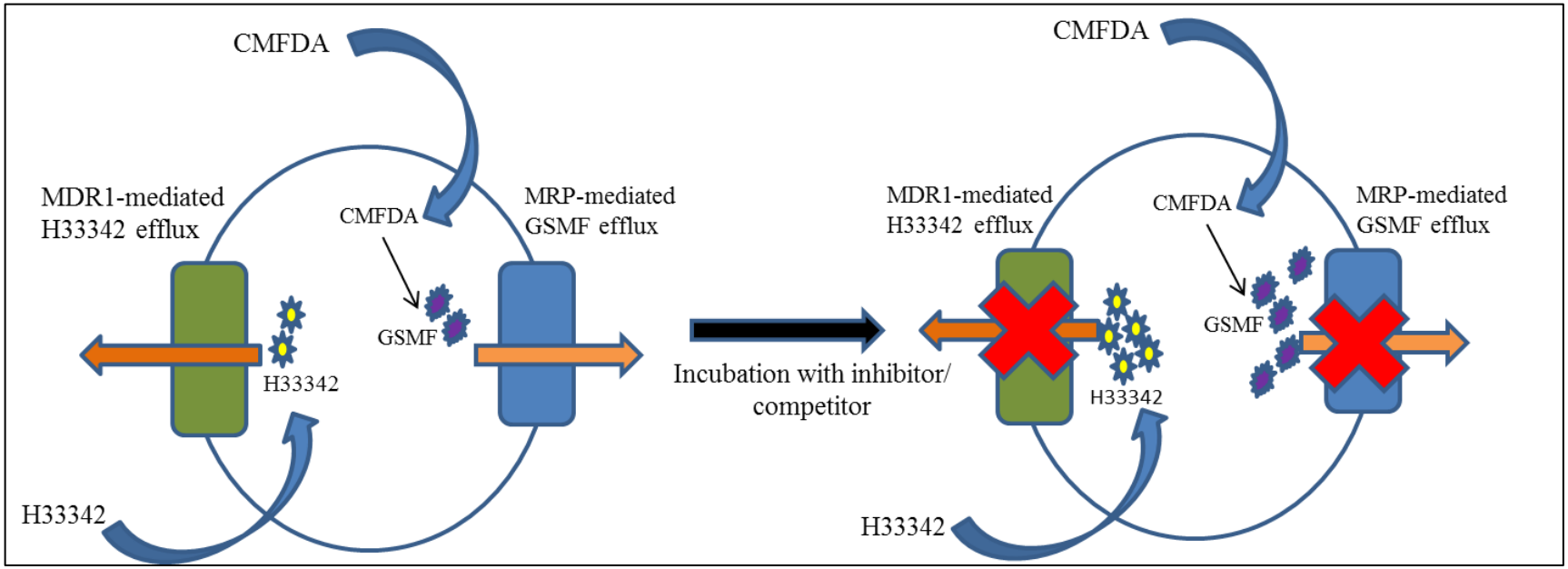


Figure 3.1 Principle of fluorescence dual dye efflux assays with substrate dyes H33342 for MDR1/mdr1 and CMFDA for MRP/mrp efflux transporters. In the absence of efflux transporters, dye diffuses through the cell membrane and establishes equilibrium. In an intracellular environment the dye becomes fluorescent, either by association with lipid (H33342; Crissman and Steinkamp, 1987) or after cleavage by intracellular esterases and subsequent conjugation with gluathione (CMFDA; Forster et al., 2008). In the presence of functional effux transporters for which it is a substrate, fluorescent dye is actively effluxed and intracellular fluorescence decreases. With the introduction of a inhibitor/competitor, efflux of dye is reduced and intracellular fluorescence rises. Observed IC_{50} values are used to measure affinity of the receptor for the competitor. H33342, Hoechst 33342; GSMF, glutathione methylfluorescein; MDR1, p-glycoprotein; MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate.

3.3.5 Protocols

Cells for transporter assays were cultured as previously described. The cells were kept at 37°C by performing the experiment on thermostatic-controlled hot plates. The cells were then washed twice with 200 µl of pre-warmed Krebs buffer (refer **section 2.2.4**). The Krebs buffer was then aspirated off from the plate and replaced with Krebs buffer containing inhibitor and incubated for 40 minutes. In the remaining Krebs-inhibitor solution, a probe substrate (H33342 and/or CMFDA) at concentration of 1 µM was added and further incubated for another 40 minutes in the absence of light. 40 minutes was the optimised time interval for transporter assays in this study, instead of 20 minutes incubation previously described by Morgan and colleagues (Morgan et al., 1989).

At the end of this period, cell monolayers were washed twice with ice cold-Krebs buffer and 200 µl fresh Krebs buffer was added to each well. Blanks wells were also prepared simulataneously using the same medium to assess background without fluorescent probe. The cellular fluorescent levels in each 96-well were measured using FLUOstar plate reader (BMG LabTech) with wavelengths of CMFDA (excitation at 480 nm, emission at 520 nm) and H33342 (excitation at 355 nm, emission at 480 nm). The fluorescence reading is correlated with the magnitude of inhibition produced by the inhibitors. To ensure that the measured differences in intracellular fluorescence were due to the test compounds rather than solvent, solution in control well contained the same concentration of solvent (DMSO or methanol) to a final solvent concentration of 0.2 %, the highest percentage of solvent applied for the tested compounds and was previously found not to affect the viability of the cells up to 48 hours treatment.

3.3.6 Data analysis for dye efflux assay

The readings from the background were subtracted from the raw data and these data were normalised to the average of readings for the solvent control, which was assigned to be 100 %. The inhibition data were then expressed as a percentage of the control. The data was then fitted with a nonlinear least-squares regression curve (Michealis-Menten), and IC_{50} and K_i values were obtained as previsouly described (see equation 2.2 in **section 2.2.6*).***

3.3.7 Statistics

All statistical analysis on data was performed using GraphPad Prism software version 4 (GraphPad Software Inc. San Diego, CA, USA) as described in **section 2.3.7**. Statistical difference between group means was tested using Student's unpaired t-test (also known as independent- samples t-test) or a One-Way ANOVA with Dunnett's post-hoc test as appropriate. Paired t-test was used when the mean of continuous variables between groups were related in some way, while two-way ANOVA was used when two independent variables were evaluated on a continuous dependent variable of interest. A value of $p < 0.05$ was considered statistically significant. Unless otherwise stated, results are expressed as the mean ± SEM.

3.4 Results

3.4.1 Expression of efflux transporter in HK-2 cells

Relative expression of efflux transporters at mRNA level

Our group has previously reported on the expression of efflux transporters in the HK-2 cells at both functional and mRNA levels (Jenkinson et al., 2012). For this novel technique two dyes in combination are used to demonstrate simultaneously the functional expression of MDR1 and MRP *in vitro*. In HK-2 cells, as shown in Figure 4.1, it was confirmed by qPCR array (cat. no: PAHS 070Z, from Qiagen Ltd., Crawley, UK) that relatively high mRNA expression of MDR1 (ABCB1) was present, but not of MRP2 (ABCC2) or BCRP (ABCG2). However, several other MRP isoforms such as MRP1,

MRP4 and to a certain degree MRP3 and MRP5 were expressed in the cells (Jenkinson et al., 2012).

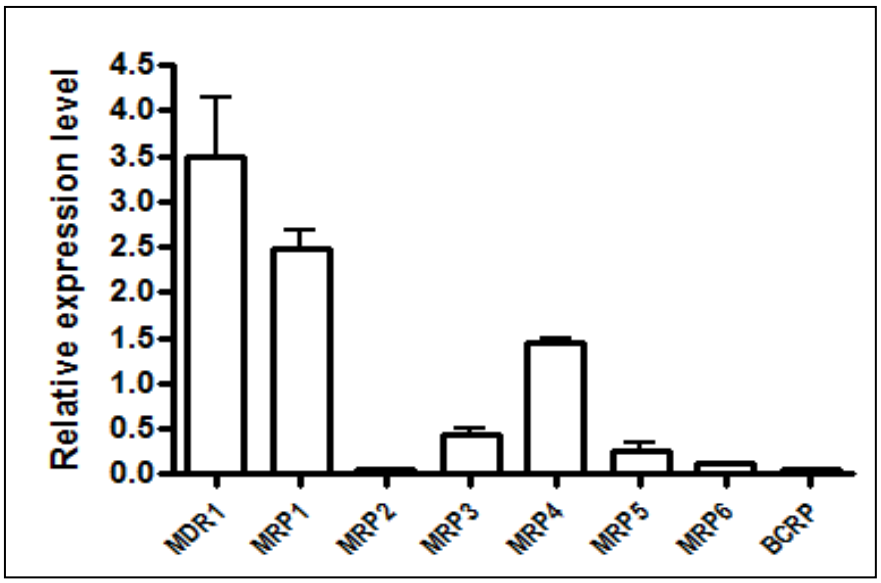


Figure 3.2 Relative expression levels of efflux transporters in HK-2 cells. The result were analysed by standard RT² Profiler PCR Array data analysis from SABiosciences website (**Section 2.2.5** in Chapter 2). The expression levels were calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to geometric mean of 5 housekeeping genes of the array. Error bars represent the mean ± SD of three independent assays of three separate cultures of HK-2 cells. MDR1, p-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein.

Optimisation of dual dye retention assay

To minimize experimental variability, functional expression of MRP and MDR1 efflux transporters in the HK-2 cells were replicated using the same batch of cells (using the same number of cell passage, but at a density of 20, 000 cells/well instead of 40,000 cells/well as was used by Jenkinson et al (2012). Consistent with the findings by Jenkinson et al (2012), I found that the expression of MDR1 and MRPs at functional level (**Figure 3.3**) could be detected by the use of CMFDA and H33342 dyes simultaneously with non-overlapping signal. Dye retention was significantly blocked by MK571 ($p<0.0001$) but was only present in a range of fluorescent wavelength specific to CMFDA, whilst blockade with CSA resulted in significant dye retention (both H33342 and CMFDA).

According to **figure 3.3a**, addition of MK571 caused significant ($P<0.0001$) increase in dye retention (from 22077 ± 5742 a.u. to 122687 ± 15057 a.u) at CMFDA wavelengths (480nm excitation, 520nm emission). Surprisingly CSA resulted in similar increase in dye retention suggesting that it could also be used as an MRP modulator. However, at H33342 wavelengths (355nm excitation, 480nm emission), MK571 did not cause any significant dye retention, whilst the dye retention was only seen by CSA addition; H33342 dye retention by CSA almost doubled when compared with wells containing the dye only (**Figure 3.3b**). Therefore, CSA was used as the MDR1 modulator at the indicated wavelength.

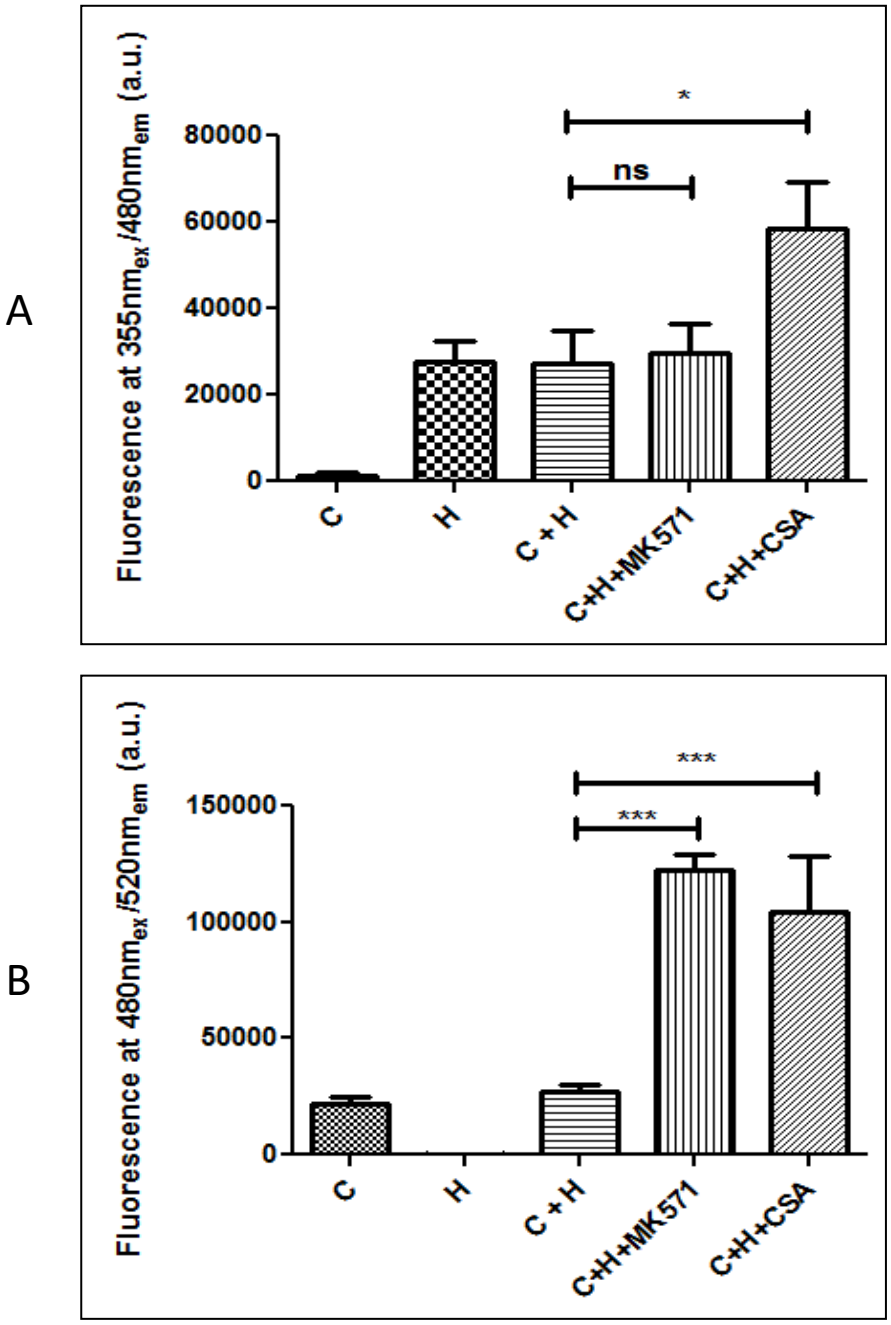


Figure 3.3 Non-overlapping fluorescent signal for CMFDA and H3334 and the specificity of MK571 as an MRP inhibitor. There was no signal for H3334 either alone or in combination with CMFDA. Data are presented as mean± SEM (n=30) from 5 determinations (6 replicates in each determination). * $p< 0.05$ *** $p< 0.0001$ ns= non-significant. C, CMFDA (5-chloromethylfluorescein-diacetate); H, Hoechst 33342; C+H, combination of both dyes; CSA, cyclosporin A; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; Em, emission; Ex, excitation.

Prior to the functional experiments, the concentration used for both dyes (i.e. 1 μM) was optimised by a series of blockade experiment using CSA and MK571 (**Figure 3.4**). The preliminary experiments in the HK-2 cells revealed a significant ($p<0.01$) concentration-dependent increase in fluorescence caused by intracellular glutathione methylfluorescein (GSMF) (the fluorescent product of the CMFDA dye as described in **section 3.3.4**) at the concentration of the CMFDA dye of 1 μM when co-incubated with 10 μM CSA. Similarly to the MDR1-mediated H33342 dye efflux, there was a significant ($p<0.0001$) concentration-dependent increase in intracellular fluorescence when H33342 (1 μM) was co-incubated with MK571 (10 μM). The efflux for both dyes (as low as 0.15 μM) was shown to be significantly inhibited (** $p<0.05$ for CMFDA and *** $p<0.001$ for H33342) in the presence of an inhibitor (at 10 μM). However, for all subsequent transporter-mediated dye efflux experiments a final concentration of 1 μM was used for both dyes to allow ready detection of dye retention.

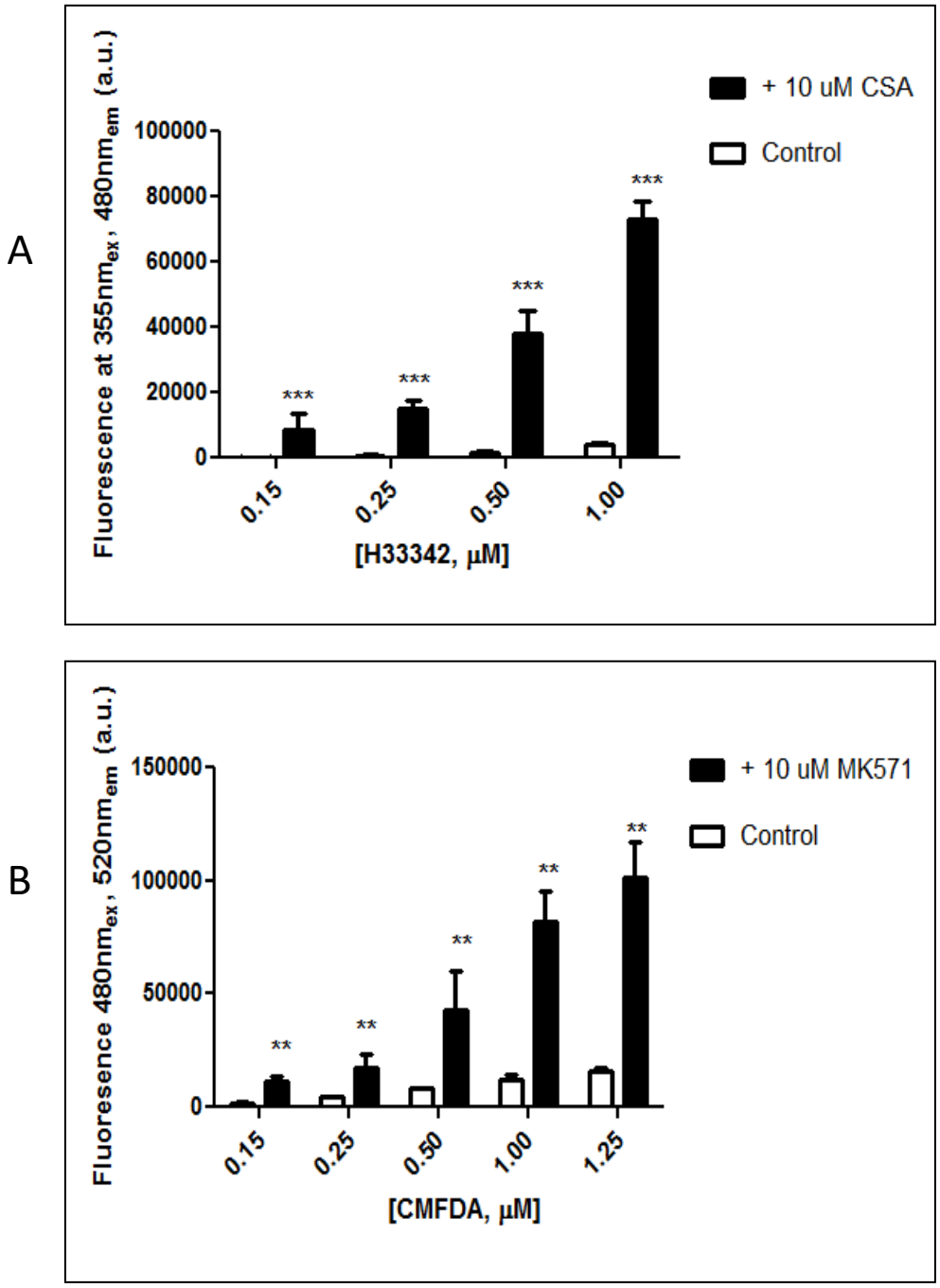


Figure 3.4 Optimisation of the concentration for the dyes used in dye retention assay in HK-2 cells. Concentration-dependent retention of (A) H33342 and (B) CMFDA dye in the absence and presence of CSA and MK571 (both inhibitors at 10 µM each), respectively. The concentration applied for the dye as low as 0.15 µM resulted in statistically significant (** $p<0.01$ for CMFDA and *** $p<0.001$ for H33342) retention in the presence of an inhibitor compared to that of control (treated with dye only). Data are represented as ± SEM (n = 18) from three independent determinations. H33342, Hoechst 33342; CMFDA, 5-chloromethylfluorescein-diacetate; CSA, cyclosporine A; Em, emission; Ex, excitation.

Next, the IC_{50} value for MK571 (**Figure 3.5**) was derived from dose-response curves relating to the ability of MK571 (0-50 μM) to increase intracellular CMFDA dye (1 μM) retention. The IC_{50} for CSA (**Figure 3.6**) was similarly gained from dose-response curves relating to the ability of CSA (0-50 μM) to increase intracellular H33342 dye (1 μM) retention. The IC_{50} values were 1.04 ± 0.4 μM and 1.36 ± 0.3 μM for MK571 and CSA, respectively. Remaining intracellular fluorescence product (exhibited by the percentage of control of the indicated dyes) of CMFDA retained by MK571 blockade was relatively six-fold higher (V_{max} = 326 ± 23 %) than that seen with H33342 dye retention after blockade with CSA (V_{max} = 58 ± 3.2 %)). This might corresponds with the relative mRNA expression level between MDR1 and MRPs as determined by the qPCR results (**Figure 3.2**).

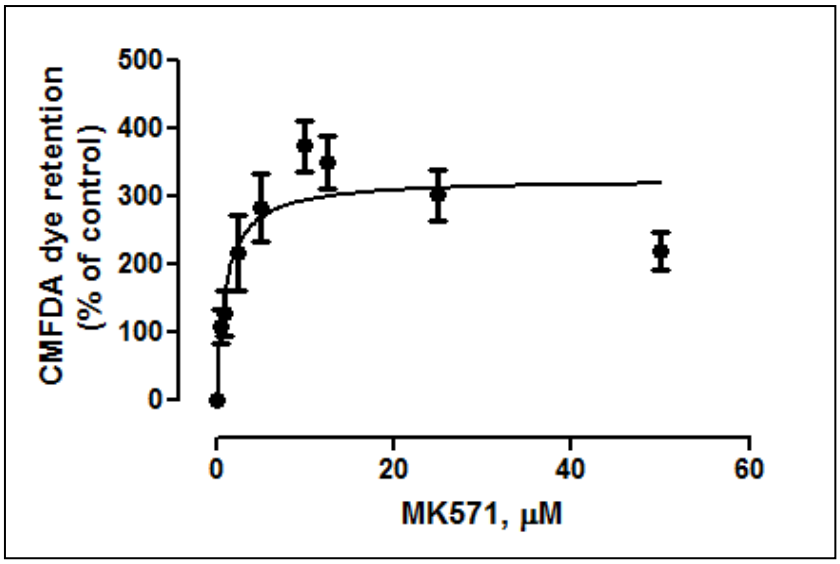


Figure 3.5 MK571 concentration-dependence assay in HK-2 cells. Kinetic analysis of the data using a least-squares non-linear regression curve fit gave an apparent IC_{50} for MK571 (normalised to dye retention in control cells treated with CMFDA only) of 1.04 ± 0.4 µM (V_{max} of 326 ± 23 %). Data are presented as a mean ± SEM (n=12) from three independent experiments. MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; CMFDA, 5-chloromethylfluorescein-diacetate.

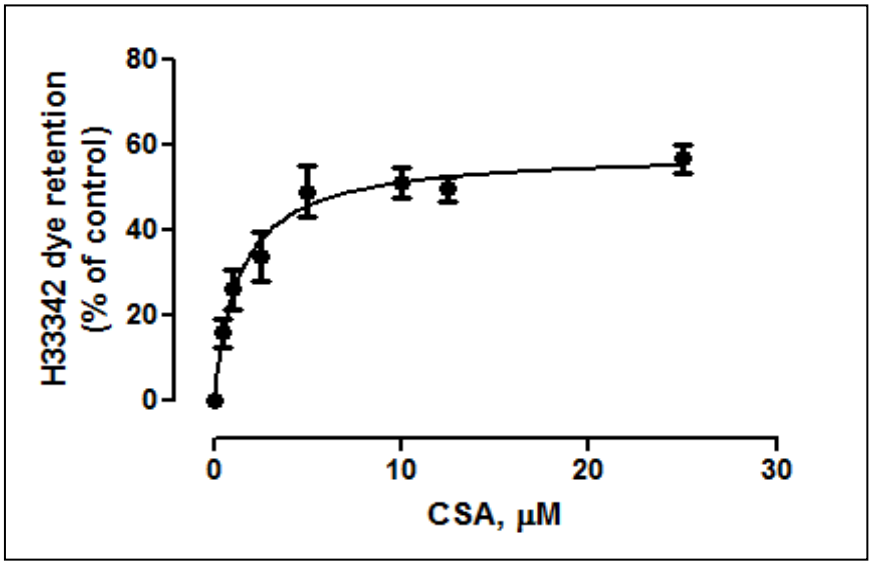


Figure 3.6 CSA concentration-dependence assay in HK-2 cells. Kinetic analysis of the data using a least-squares non-linear regression curve fit gave an apparent IC_{50} for CSA (normalised to dye retention in control cells treated with H33342 only) of 1.36 ± 0.3 µM (V_{max} of 58 ± 3.2 %). Data are presented as a mean ± SEM (n=18) from three independent experiments. CSA, Cyclosporine A; H33342, Hoechst 33342.

The impact of statins on the MRP-mediated CMFDA efflux

As an initial experiment to determine the impact of statins on functional expression of MRPs, inhibitory magnitude shown by all 4 tested statins (all at 10 µM) was compared to that of MK571 and CSA (both also at 10 µM). Of the 4 statins examined, as shown in **figure 3.7**, simvastatin and atorvastatin (although more than 3-fold lower in CMFDA dye retention than that produced by MK571) significantly (***$p<0.001$) inhibited CMFDA dye efflux. In contrast, pravastatin and rosuvastatin had no significant effect on CMFDA dye retention when the cells were pre-treated with both the hydrophilic statins. Interestingly, CSA was also found to significantly (***$p<0.001$) inhibit CMFDA dye efflux, suggesting that it also has affinity towards MRPs. Prior experiments showed that the solvents used (i.e., 100 % methanol for simvastatin, while DMSO for others) for the dissolution of the statins did not impact the results as they had no effect on fluorescence measurements when compared to those from control samples which contained only CMFDA or H33342 (data not shown).

The concentration for both simvastatin and atorvastatin to inhibit 50% of maximal velocity of the CMFDA dye retention (IC_{50}) was obtained by incubating the cells with each statin (0-200 µM) (**Figure 3.8**). It was shown that simvastatin exhibited higher affinity (lower IC_{50} value) toward MRP-mediated CMFDA efflux than that of atorvastatin (86.3 ± 7.9 µM versus 151.7 ± 40.3 µM). Neither pravastatin, nor rosuvastatin inhibited CMFDA retention

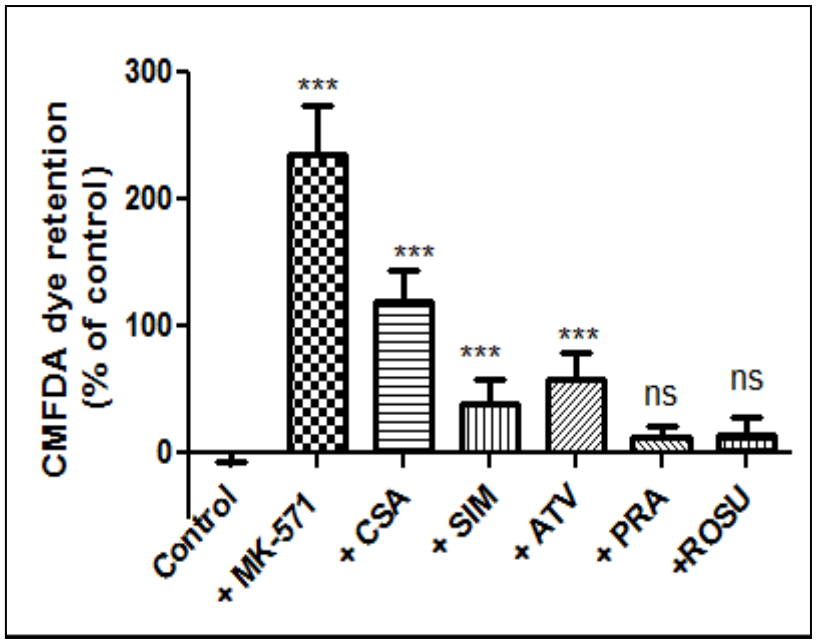


Figure 3.7 The inhibition of MRP-mediated CMFDA dye efflux by inhibitors (10 µM) in HK-2 cells. Data are presented as a mean ± SD (n=18) from 3 independent experiments. Data are analysed with One-way ANOVA and Dunnett's post test; ***P<0.0001 and ns (non-significant) versus control. MRP, multidrug resistance-associated protein; CSA, cyclosporine A; SIM, simvastatin; ATV, atorvastatin; PRA, pravastatin; ROSU, rosuvastatin; CMFDA, 5-chloromethylfluorescein-diacetate; MK-571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid.

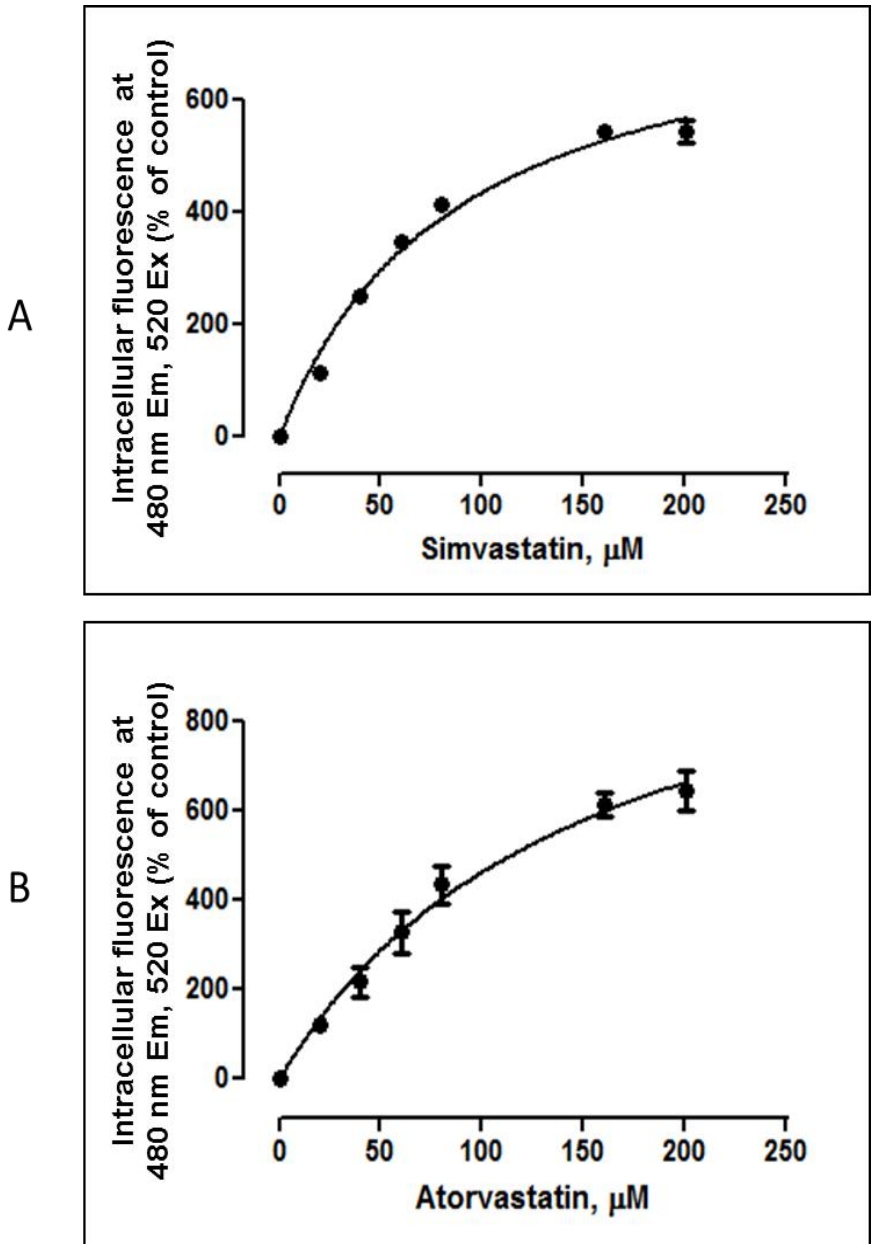


Figure 3.8 Dose-response curve for the ability of simavastatin (A) and atorvastatin (B) to inhibit MRP-mediated CMFDA efflux. Data are mean ± S.E.M. n = 18 wells per concentration from a single experiment, representative of three independent experiments. Em, emission; Ex, excitation; MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate.

The impact of statins on the MDR1-mediated Hoechst 33342 dye efflux

Since there was mRNA evidence for MDR1 transporter in HK-2 cells (**Figure 3.2**), it was decided to determine functional expression of the transporter in this cell line by determining H33342 dye retention**. Figure 3.9** compares the inhibitory magnitude of all four tested statins to that of CSA, the MDR1 modulator. In contrast to the significant inhibitory effect of simvastatin and atorvastatin onto the MRP-mediated CMFDA efflux, they did not significantly inhibit the MDR1-mediated H33342 dye efflux at the same concentration (i.e., 10 µM) as CSA did.

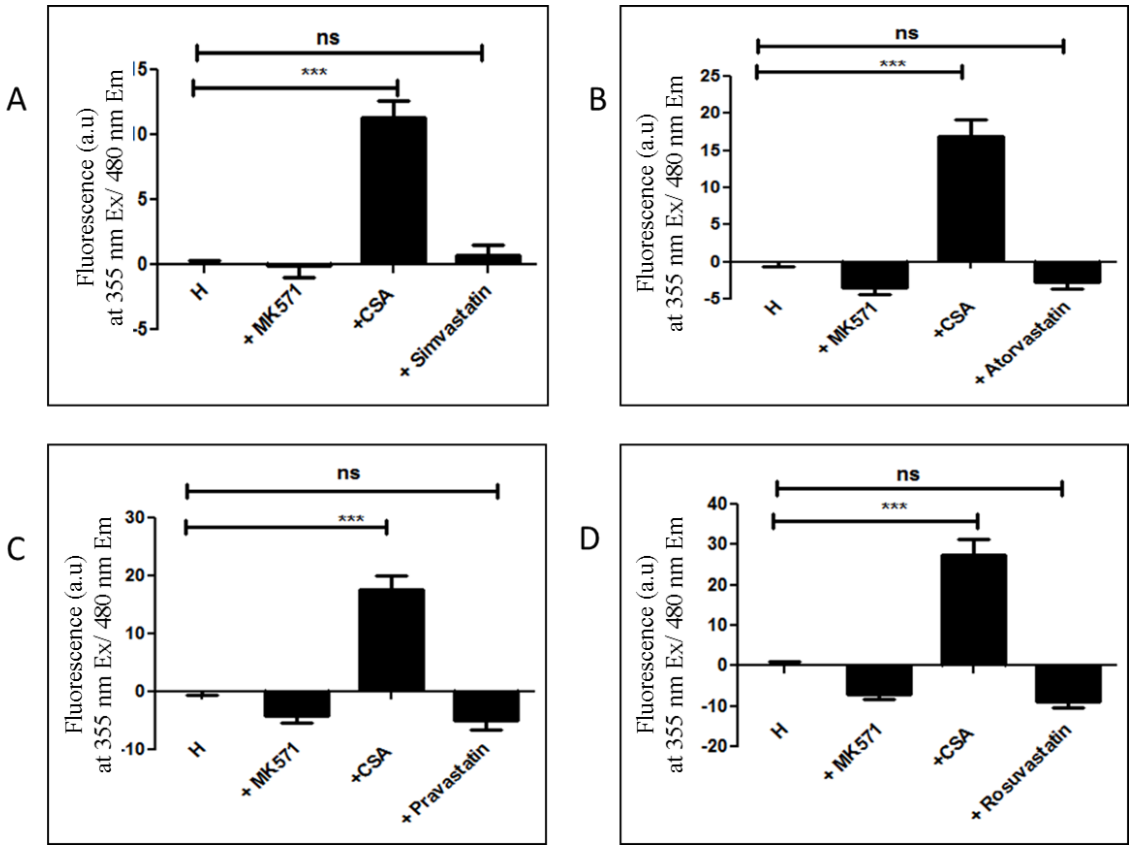


Figure 3.9 The impact of statins on MDR1 function in comparison with CSA. The inhibition of H3342 dye (H) efflux by simvastatin (A), atorvastatin (B), pravastatin (C) and rosuvastatin (D). Data are presented as mean ± SEM (n=18) from three independent experiments. Data were analysed with One-way ANOVA and Dunnett's post test; ***P<0.001 and ns (non-significant) versus control. MDR1, p-glycoprotein; CSA, cyclosporine A; H, Hoechst 33342; Em, emission; Ex, excitation; a.u, arbitrary unit.

3.4.2 Expression of efflux transporter in L6 rat skeletal muscle cells

Regulation of efflux transporters in L6 cells

The expression of a series of efflux transporters (both SLC and SLCO sub-groups) in the L6 cells was carried out by qPCR array (catalogue no: PARN 070Z from Qiagen Ltd., Crawley, UK). The experiment was performed using total RNA isolated from L6 cells at myotube stage on day 7 after differentiation. To assess the effect of statins on gene expression of these efflux transporters in L6 myotubes, the cells were pre-treated with simvastatin (2µM) for 48 hrs prior to RNA extraction (day 5) and compared to untreated control cells. Among the ABC efflux transporters, only mRNA of Mrp1 (Abcc1) was found to be highly expressed in L6 cells compared to the other Mrps; Mrp2-Mrp6 (**Figure 3.10**). It seems that the regulation of Mrp1 had not been much affected by pre-treatment with simvastatin. Although the expression of some transporters, for example Mrp2, Mrp3, Mrp6 and Bcrp1, was lower with simvastatin pre-treatment, the relative expression level was too low (i.e., < 0.02) to infer the effect of the statin treatment on these transporters. In addition, supported by high crossing point (C_p) values i.e., representing the PCR cycle number at which the reporter dye fluorescence was detectable above the background fluorescence, with the cut-of-point of 33, the expression of Mrp2-Mrp6, Mdr1, Mdr2 and Bcrp1 were considered low in the L6 cells with mean C_p values in the range of 29-31 (**Appendix E**).

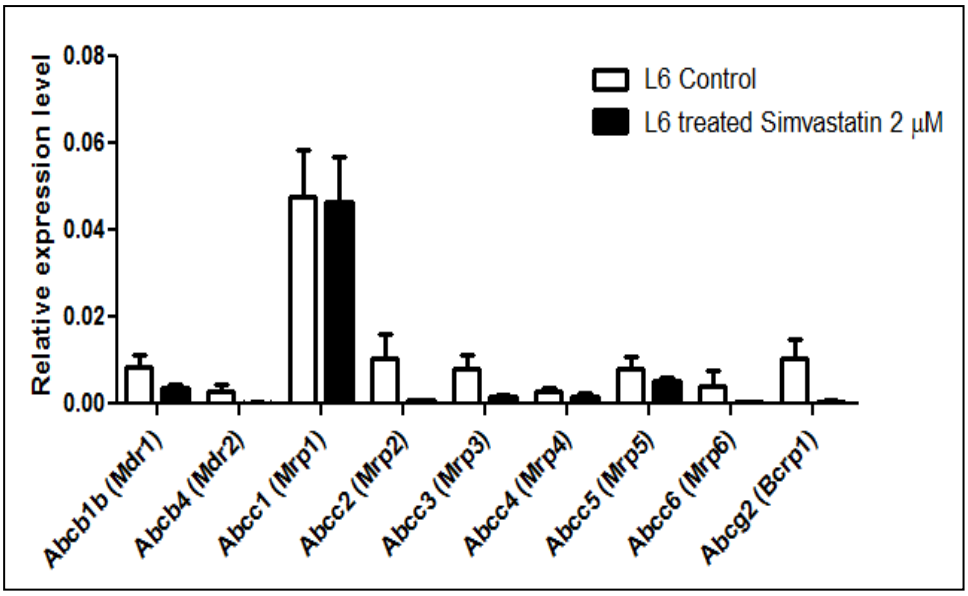


Figure 3.10 A selection of key efflux transporters in cDNA of L6 myotubes analysed by drug transporters qPCR array. The L6 myotubes were differentiated for 7 days and treated with simvastatin (2 µM) for 48 hours prior to RNA extraction. The corresponding culture with vehicle solvent (MeOH, 0.02 % v/v) represented the control. The relative expression level was analysed by standard qPCR Array and data analysis from SABiosciences website (http://www.sabiosciences.com/pcrarraydataanalysis.php). The expression levels were calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to the geometric mean of five housekeeping genes provided by the array. Error bars represent the mean ± SEM (n=3). Abc, ATP-binding cassette; Mdr1, p-glycoprotein; Mrp, multidrug resistance-associated protein; Bcrp, breast cancer resistance protein.

CMFDA dye retention assay

Owing to the evidence of relatively high mRNA expression of MRPs, but not MDR1, in L6 cells (**Figure 3.10**), it was decided that the impact of statin on functional expression of efflux transporters will be studied focusing only on the MRP-mediated CMFDA efflux assay. The concentration used for the dye (i.e. 1 µM), as in MRP functional experiments in HK-2 cells, was pre-determined by a series of blockade experiment with MK571 (5 µM). Similar to the findings in the HK-2 cells, L6 myotubes were shown to exhibit high functional expression of MRP transporters. The results indicated that the lowest dye's concentration (i.e., at 0.15 µM), efflux was significantly (*** $p<0.001$) inhibited in the presence of MK571 (5 µM) compared to control wells that were treated with dye only (**Figure 3.11**). The concentration –dependence of the CMFDA after blockade with MK571 (5 µM) resulted in K_m value of 1.43 ± 0.4 µM.

In order to show that the functional expression of MRPs could be affected by statin, L6 myoutubes were pre-treated with simvastatin (2 µM) up to 48 hours prior to their use in the functional assay. Compared to the un-treated L6 myotubes, there was no obvious up-regulation or down-regulation of the MRPs at functional levels. The IC_{50} and V_{max} values for MK571 (0-50 µM) were similar for both treated (IC_{50} = 0.81 ± 0.2 µM, V_{max} = 219 ± 11 %) and un-treated (IC_{50} = 0.91 ± 0.1 µM, V_{max} = 246 ± 7.4 %) cells as shown in **Figure 3.12**.

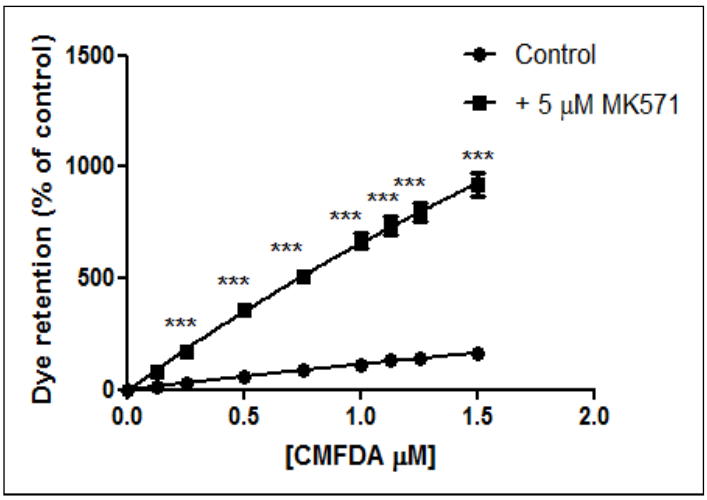


Figure 3.11 Inhibition of MRP-mediated CMFDA efflux by MK571 in L6 cells. Addition of MK571 at 5 µM resulted in a significant increase in CMFDA dye retention compared to that of control wells with K_m value of 6.66 ± 3.6 µM. ***P<0.0001 demonstrates the level of significance compared to the control without MK571 pre-treatment (paired T-test).MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid.

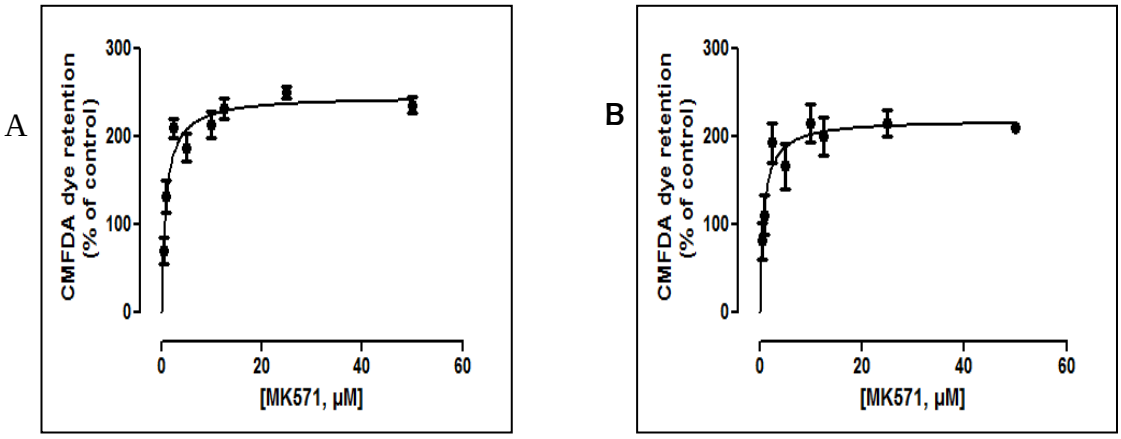


Figure 3.12 Dose-response curve for MRP-mediated CMFDA (1 µM) efflux inhibition by MK571 in L6 cells. L6 myotubes were exposed with simvastatin (**A**) and without simvastatin (**B**) pre-treatment for 48 hrs. Dye retention was measured after treatment with a range of MK571 concentrations after 40 minutes. Each point represents mean ± SEM (n = 18) from 3 independent experiments. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid.

The impact of statins on the MRP-mediated CMFDA efflux

Having established the optimisation of MRP functional assay in L6 myotubes, the impact of statins to retain intracellular CMFDA dye (i.e GSMF) was compared to that of MK571. Of the four statins tested (each at 10 µM), only simvastatin (although five-fold lower than that of MK571) resulted in significant ($p<0.05$) CMFDA dye retention (**Figure 3.13**). Interestingly, having comparing dose-response curve between simvastatin (**Figure 3.14a**) and atorvastatin (**Figure 3.14b**), atorvastatin also significantly ($P<0.0001$) resulted in CMFDA dye retention at the concentration higher than 25 µM. Whilst, a rather gradual increase in CMFDA dye retention was seen with simvastatin treatment at 0-50 µM with the IC_{50} value of 25.05 ± 9.1 (95% CI= 12.93 – 37.40) (**Figure 3.15**). Also, as expected, both the hydrophilic statins; pravastatin (**Figure 3.14c**) and rosuvastatin (**Figure 3.14d**), did not significantly affect dye retention up to the highest final drug concentration of 50 µM.

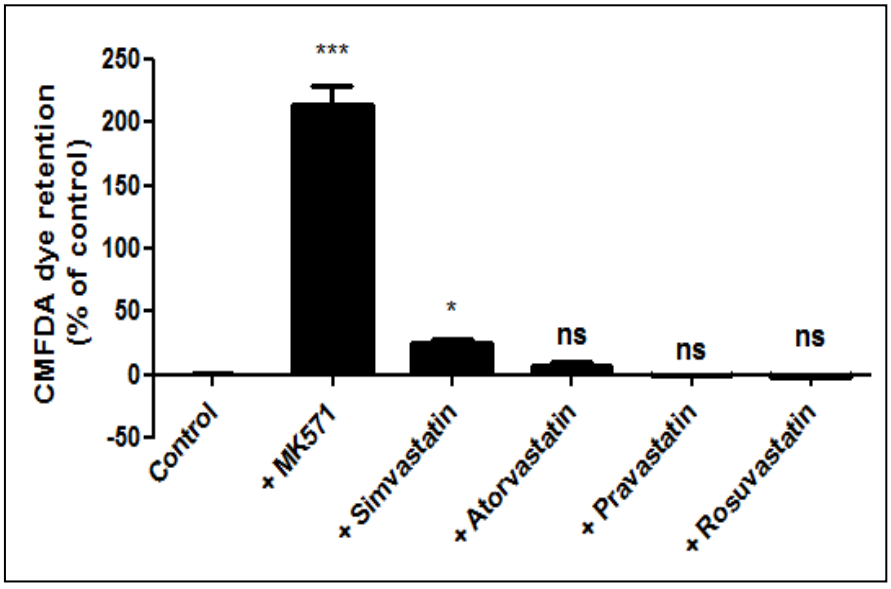


Figure 3.13 The inhibition by statins of the MRP-mediated CMFDA dye efflux in **L6 cells**. The concentration of all inhibitors used were 10 µM. Data are presented as mean ± SD (n=18) from 3 independent experiments. Data were analysed using one-way ANOVA and Dunnett's post test; *P<0.05 and ns (non-significant) versus control. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; ns, non significant.

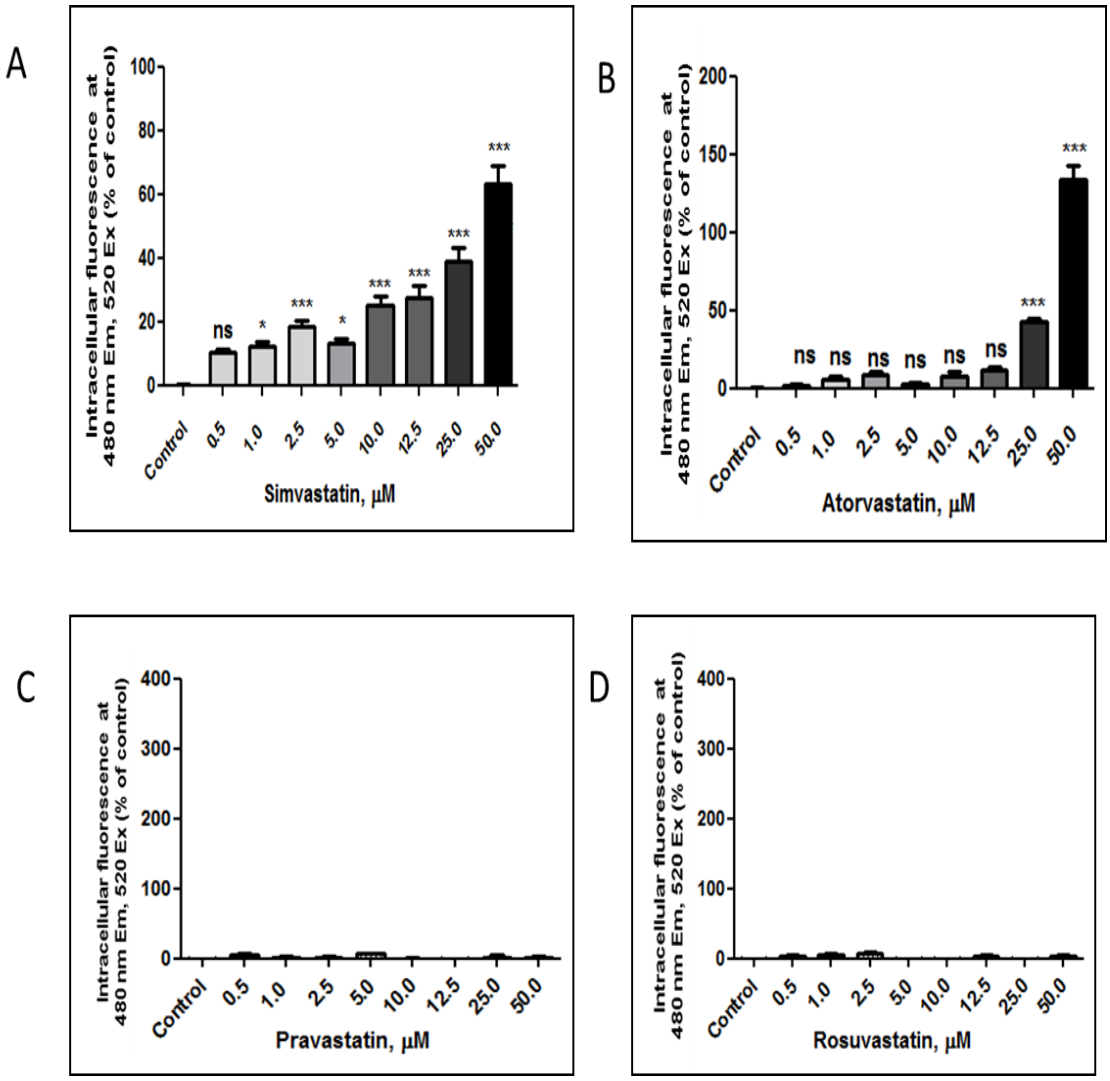


Figure 3.14 Effect of statins (0.5-50 µM) on the MRP-mediated efflux of CMFDA in L6 cells. Significant dye retention was noted for simvastatin and atorvastatin at concentrations as low as 1 µM and 25 µM, respectively. Data are presented as mean ±S.E.M. n = 6 wells per drug concentration from a single experiment representative of 4 independent experiments, *** P<0.0001, *P<0.05 and ns (non-significant) compared to the control (without statins treatment). Data analysed using one-way ANOVA and Dunnett's post-test. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; Em, emission; Ex, excitation.

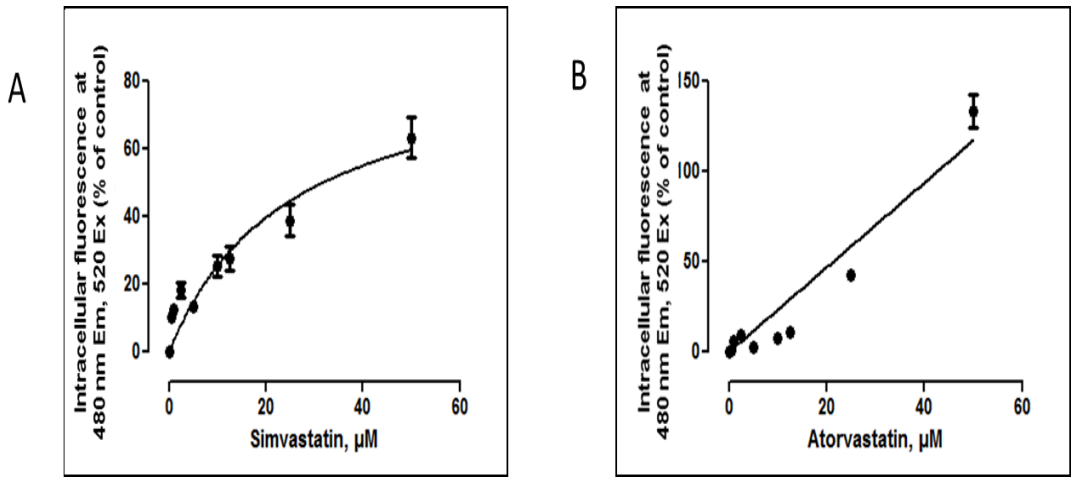


Figure 3.15 Dose-response curve for simvastatin (A) and atorvastatin (B) to inhibit MRP-mediated efflux of CMFDA in L6 myotubes. Data are presented as mean ± S.E.M (n = 18), with 6 wells per concentration, from 3 independent experiments. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; Em, emission; Ex, excitation.

3.4.3 Expression of efflux transporter in human muscle cells

Relative expression of efflux transporters at mRNA level

The expression of a series of efflux transporters (both SLC and SLCO sub-groups) in human muscle myotubes was assessed using qPCR array (catalogue no: PAHS 070Z, from Qiagen Ltd. Crawley, UK). The experiment was performed using total RNA isolated from the cells at myotube stage on day 7 after differentiation. Among the ABC efflux transporters, only mRNA of MRP1 (ABCC1) was found to be highly expressed, though it was less than half the expression level found in L6 myotubes. MRP3 (ABCC3), MRP4 (ABCC4) and to some degree BCRP (ABCG2) were also present at low level of mRNA expression (**Figure 3.16)**.

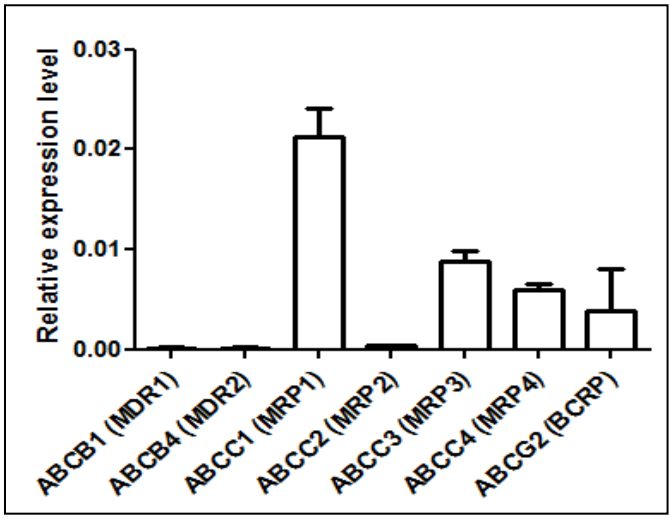


Figure 3.16 A selection of key efflux transporters in cDNA of human muscle myotubes analysed by drug transporters qPCR array. The human muscle cells at myoblast stage were differentiated up to 7 days prior to RNA extraction. The relative expression level was analysed by standard qPCR Array and data analysis from SABiosciences website (http://www.sabiosciences.com/pcrarraydataanalysis.php). The expression levels were calculated using the $2^{-\Delta\Delta Cp}$ method and are relative to the geometric mean of 5 housekeeping genes provided by the array. Error bars represent the mean ± SD (n=2). ABC, ATP-binding cassette; MDR1, p-glycoprotein; MDR2, multidrug resistance protein 2; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein.

CMFDA dye retention assay

Owing to the evidence of relatively high mRNA expression of MRP1, but not other MRP sub-types as well as MDR1 (ABCB1) and BCRP (ABCG2), in human muscle myotubes (**Figure 3.16**), it was decided that that studying the impact of statin impact on functional expression of efflux transporters in this cell line will be focused on MRP-mediated CMFDA efflux assay. In addition, in order to confirm the qPCR array results, a preliminary experiment for MDR1- and/or BCRP-mediated H33342 dye efflux was performed in the presence of CSA (5 µM) and K0143 (1 µM), MDR1 inhibitor and BCRP inhibitor, respectively. As expected, there was no significant H33342 dye retention using both inhibitors when compared to that of control (data not shown) suggesting the absence of no functional expression of either MDR1 or BCRP transporters in this cell line.

MRP-mediated CMFDA efflux was inhibited in the presence of MK571 (at 5 µM) having lower IC_{50} value (1.17 ± 0.3 µM, **Figure 3.17a)** thus suggesting higher affinity, than that of control without MK571 (5.19 ± 1.6 µM, **Figure 3.17b**). Intriguingly, the measurement of intracellular CMFDA fluorescence at the indicated wavelength was still detected for control wells without MK571 (although in the absence of MK571 which suggest poor MRP-mediated CMFDA efflux rate in these cells. Also, in comparison to other cell lines studied; HK-2 and L6 cells, the percentage of the CMFDA dye retained in the cells after blockade with MK571 (5µM), presented by the V_{max} value, was lower (approximately 15 – 30 % of control) consistent with low expression level of MRP transporters in the human myotubes.

The impact of simvastatin (1 µM) pre-treatment on the functional expression of MRPs was evaluated. As shown by **Figure 3.18** simvastatin pre-treatment on human muscle did not result in significant CMFDA dye retention compared to control suggesting that simvastatin did not modify MRP function.

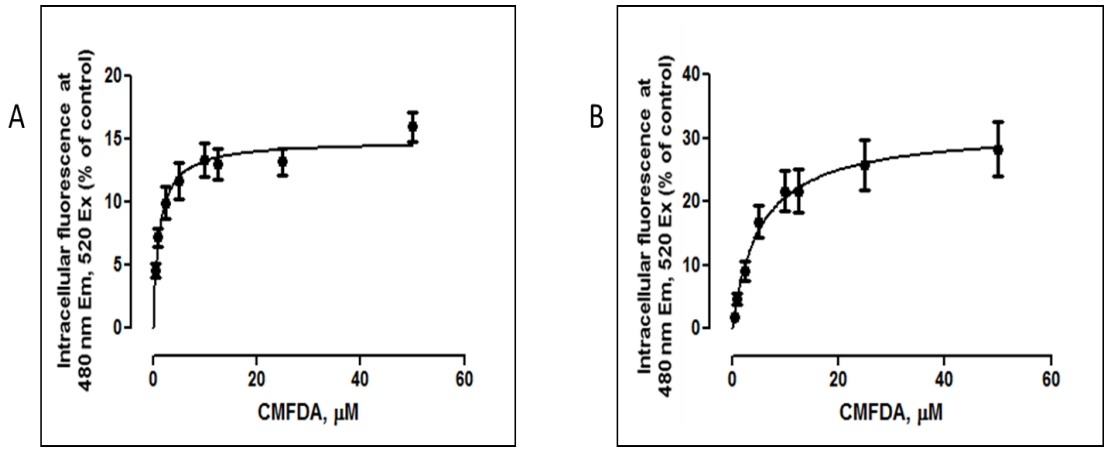


Figure 3.17 MRP-mediated CMFDA efflux inhibition by MK571 in human myotubes with (A) and without (B) the presence of MK571. Data are mean ± S.E.M (n = 18), with 6 wells per concentration, from 3 independent experiments. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; Em, emission; Ex, excitation.

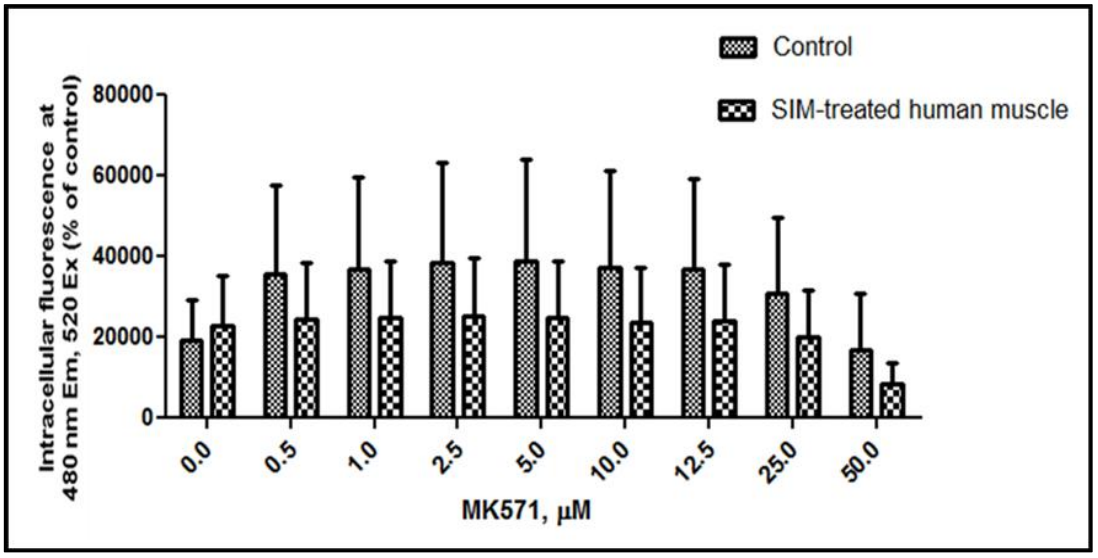


Figure 3.18 The impact of simvastatin (1 µM) pre-treatment on MRP-mediated CMFDA efflux in human myotubes. There was no significant difference in CMFDA intracellular fluorescence (two-way ANOVA) between the simvastatin-treated human muscle cells after treatment with MK571 (0-50 µM) and the control. Data are presented as mean ± SD (n = 18), with 6 wells per concentration, from three independent experiments. SIM, simvastatin; MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; Em, emission; Ex, excitation.

The impact of statins on the MRP-mediated CMFDA efflux

As with other studied cell lines (**section 3.4.1** and **section 3.4.2**), the ability of statins to retain intracellular CMFDA dye (i.e GSMF) was compared to that of MK571. Of the four statins tested (each at 10µM), only simvastatin significantly inhibited ($p<0.01$) CMFDA dye efflux (**Figure 3.19**). Interestingly, having compared dose-response effect for all the four statins (0 – 50 µM), a gradual increase in CMFDA dye retention was also noted with atorvastatin and pravastatin, though to a significantly lower extent than that of simvastatin (**Figure 3.20**). The IC_{50} values of the inhibitors are 0.06 ± 0.13 µM (V_{max} = 8.5 ± 0.9 %), 0.50 ± 1.48 µM (V_{max} = 7.0 ± 1.7 %), 3.19 ± 1.22 µM (V_{max} = 3.8 ± 0.4 %%) and 1.14 ± 1.11 µM (V_{max} = 6.6 ± 1.2 %) for MK571, simvastatin, atorvastatin and pravastatin, respectively (**Figure 3.21**). Lastly, of the four statins tested, only rosuvastatin (up to a final concentration of 50 µM) was devoid of any inhibitory activity. According to the IC_{50} values, MK571 had the highest affinity for MRP-mediated CMFDA efflux in human muscle cells followed simvastatin > pravastatin > atorvastatin > rosuvastatin. It could be that specific rosuvastatin's affinity to MRP2 transporter (Jemnitz et al., 2010), for which it was not observed at the mRNA levels in this cell line (**Figure 3.16**), resulted in lack of CMFDA dye retention with rosuvastatin treatment in **figure 3.19** and **figure 3.20**.

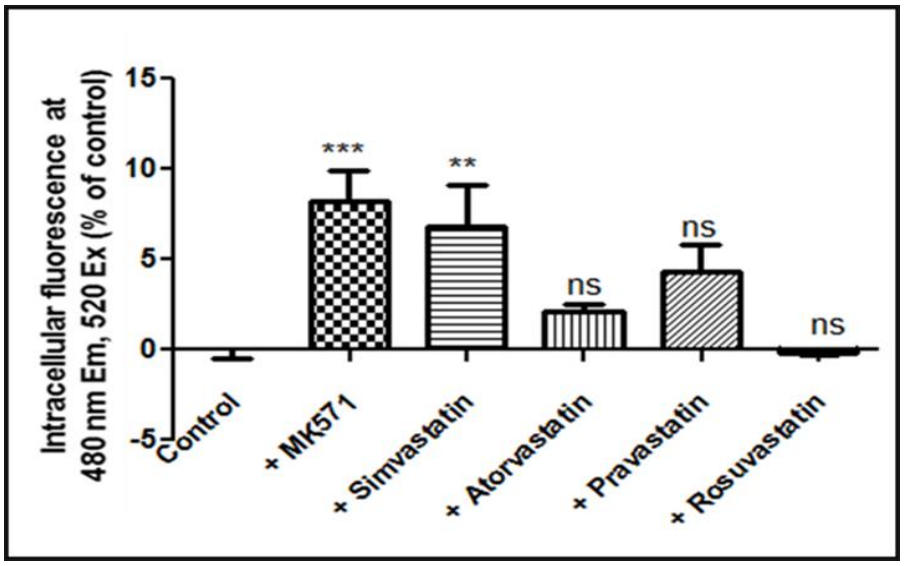


Figure 3.19 The inhibition of MRP-mediated CMFDA dye efflux by MK571 and statins in human myotubes. Data are presented as mean ± SEM (n=18) from 3 independent experiments. Data were analysed with one-way ANOVA and Dunnett's post test; ***P<0.0001, **P<0.01 and ns (non-significant). MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; Em, emission; Ex, excitation.

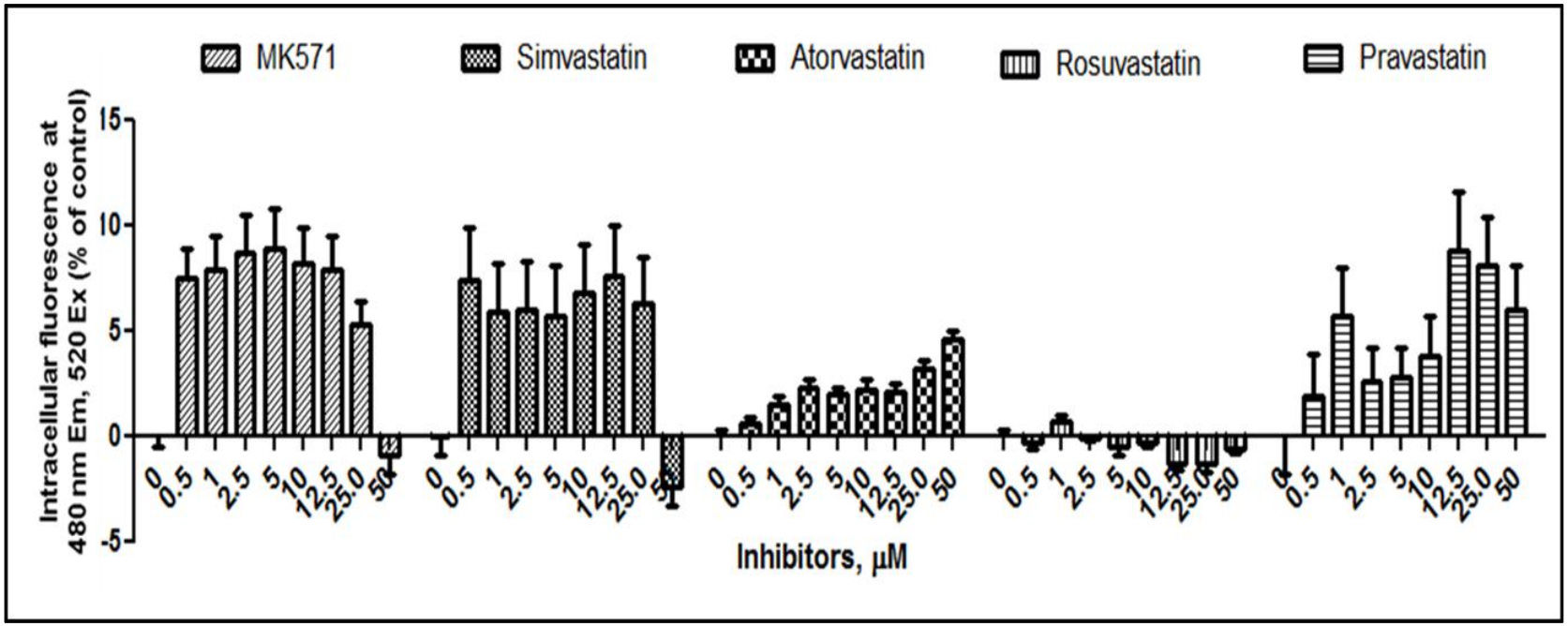


Figure 3.20 The dose-response effect of statins on MRP-mediated CMFDA efflux in comparison with MK571. The result presents CMFDA-dye retention (% after controlling for dye-only wells) after statin treatment for 40 mins in human muscle cells. Data are shown as mean ± SD (n=12) from two independent experiments. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid; Em, emission; Ex, excitation.

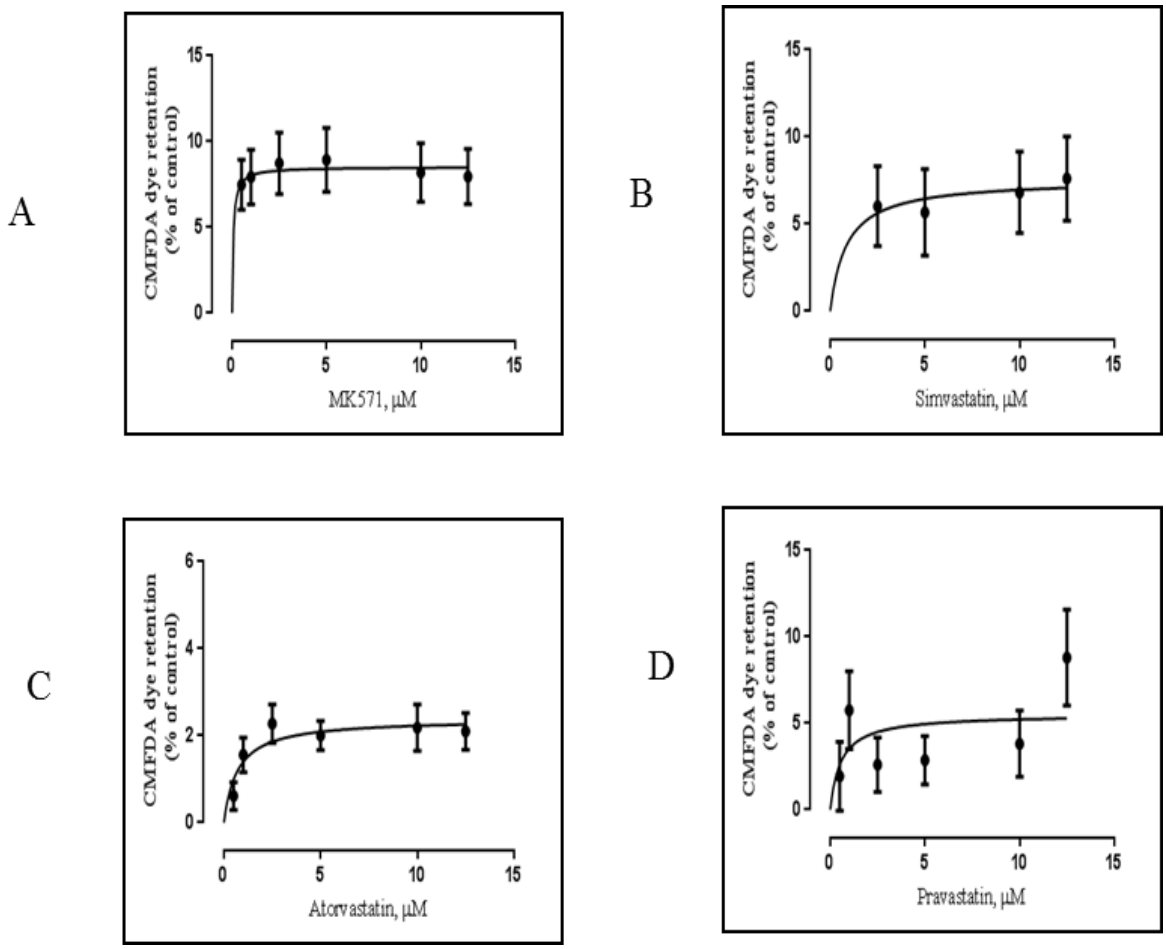


Figure 3.21 Dose-response curves for MK571 (A), simvastatin (B), atorvastatin (C) and pravastatin (D) for inhibition of MRP-mediated CMFDA efflux in human muscle cells. Data are presented as mean ± S.E.M (n = 18), with 6 wells per concentration, from three independent experiments. MRP, multidrug resistance-associated protein; CMFDA, 5-chloromethylfluorescein-diacetate; MK571, 5-(3-(2-(7-Chloroquinolon-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid.

3.5 Discussion

The main aim of this study was to determine the impact of statins on the functional expression of efflux transporters of the ATP-binding cassette (ABC) family including Multidrug Resistance-associated Proteins (MRPs) and Multidrug resistance proteins (MDR1 also known as P-glycoprotein) using an *in vitro* cell-based system. Unlike the OATP1b1 uptake transporter, limited information is available on the impact of statins on the functional expression of the ABC efflux transporters and the subsequent effect on statin pharmacokinetics and exposure in skeletal muscle cells.

Although in its infancy, pharmacogenetic studies on the impact of inter-individual variability of ABC efflux transporters in determining statin efficacy and safety, are of interest to many researchers at the present time (Giacomini et al., 2013). Preliminary work on genetic polymorphism affecting liver efflux transporters ABCG2 and ABCG8 in particular, has been reported to affect LDL-C lowering capacity of both simvastatin and atorvastatin (Hu et al., 2011, Kajinami et al., 2004a). Both studies have shown that carrier of transporter's variants in both genes were associated with a greater LDL-C reduction compared to the wild-type. Specifically, Kajinami and colleagues (Kajinami et al., 2004a) demonstrated that atorvastatin, although at low dose (10mg), was associated with LDL-C reduction for about 3 % in ABCG8 mutants. Although it is still crucial to fully describe the association between polymorphism in the efflux transporters and statin response, as the LDL-C reduction capacity reported was borderline-significant ($p=0.048$) and no subsequent replication studies have been done on the characterised polymorphism. MRP efflux transporters however, are also considered among others of the best-characterised genetic factors to be associated with statin concentration in skeletal muscle (Canestaro et al., 2014, Rodrigues, 2010).

Furthermore, preliminary findings at cellular level have provided convincing evidence that statins have an effect on efflux transporters, particularly MRPs, and that efflux transporters may be involved in statin-related myotoxicity (SRM) (Rodrigues, 2010, Knauer et al., 2010, Dorajoo et al., 2008). It has been proposed that reduced statin hepatobiliary excretion due to the inhibition of efflux transporters results in altered statin disposition and enhanced drug toxicity. However, further studies will be needed to verify such possibility (Rodrigues, 2010). Numerous *in vitro* studies targeting hepatocytes as the model to demonstrate statin uptake and efflux have been reported (Imaoka et al., 2013,

Pfeifer et al., 2013, Jemnitz et al., 2010, Ishigami et al., 1995). However research identifying muscle membrane transporters as determinants for statin-related side-effects is thought to be more relevant as muscle toxicity is a more common side-effect [OR 2.63 (95% CI 1.50 to 4.61)] (Macedo et al., 2014b).

In this study, using the same assay design as previously described (Jenkinson et al., 2012), functional expression of efflux transporters was assessed in muscle cells from both rat and human, along with a replication study in the HK-2 cells. Basically, the working principles for this functional assays are based on the concept that inhibition of transporter-mediated probe substrate (i.e fluorescent dye) efflux by an inhibitor (modulator that irreversibly binds and inactivates functional activity of the transporter), is an indirect measure of affinity to the transporter as discussed in section 2.6. Before conducting the efflux assay using statins as the modulator, an optimised test for probe substrates that have an affinity towards the studied transporters were elucidated. Two dyes, namely CMFDA and H33342, have previously been used as the probe substrates and the former dye easily permeates into the cell membrane to produce fluorescent substrate whilst the latter dye binds with cell DNA in order to become fluorescent (Hooijberg et al., 2004). The fluorescent substance of the CMFDA dye (GSMF) is specifically excreted by MRP efflux transporter, and the retention of the intracellular substance (depicted by fluorescent intensity produced) directly indicates inhibition capacity of an inhibitor of the transporter. In this study, I confirmed that both MK571 and CSA inhibited the MRPs transporter at the CMFDA wavelengths. The same working principle applies to the efflux inhibition of MDR1-mediated H33342 dye by CSA, but not MK571, suggesting that CSA can be used as an inhibitor for both MRP and MDR1 transporters. I further confirmed (**Figure 3.3**) the presence of both MDR1 (characterized by H33342 dye retention) and MRPs (CMFDA dye retention) in HK-2 cells. Moreover, consistent with our previous published study, I was able to demonstrate the presence of CMFDA, either alone or in combination with H33342, and vice versa, which did not overlap with each other's fluorescence signal, in HK-2 cells, suggesting their specific affinity to the studied transporters.

The functional expression of efflux transporter in this study is likely to have been attributed to by MRP1 owing to its relatively high expression level in all three cell lines studied. MRP1 is expressed ubiquitously, and is localised to the basolateral, rather than apical, membrane of epithelial cells. As with MRP2, MRP1 primarily effluxes a wide

range of substrates (Ballatori et al., 2005, Leslie et al., 2001), and may act as the most important efflux transporter for the extrusion of toxins or metabolites from cellular metabolism as suggested by Mueller and colleagues (Mueller et al., 2005). Among MRPs, although less evidence is available on the capacity of MRP1 for statin efflux than that demonstrated by MRP2 (Ellis et al., 2013), it has been demonstrated that polymorphism in both ABCC1 (MRP1) and ABCC2 (MRP2) genes are equally involved in the incidence of statin resistance and response (i.e. patients fail to achieve adequate reduction of LDL-C level) (Reiner, 2014, Rodrigues, 2010). Since MRP2, the 190 kDa membrane glycoprotein, is highly expressed in human apical hepatocytes (Buchler et al., 1996, Konig et al., 1999), the mutation and/or inhibition of MRP2 may become a major determinant of biliary excretion of statins resulting in statin plasma elevation, a risk factor for statin-related myotoxicity. Nevertheless, at local exposure in skeletal muscle cells, it has been shown that both atorvastatin and rosuvastatin accumulation is reduced due to MRP1 over-expression (Knauer et al., 2010) suggesting that the efflux of both statins is also attributed to the MRP1 transporter which is consistent with that found by Dorajoo et al. (2008).

I was able to demonstrate that simvastatin had affinity to MRPs, though to a lower extent than that of MK571, based upon the inhibition of MRP-mediated CMFDA efflux in both rat and human muscle. It should be kept in mind that the affinity of simvastatin towards MRP-mediated CMFDA efflux does not distinguish between MRP1 substrate and inhibitor directly, but it is rather a modulator for MRP1 function since MRP1 is expressed at the highest mRNA level among other MRPs in all three cell lines studied. This observation warrants further evaluations possibly by direct transport study using radiolabelled simvastatin.

The study results provide evidence that statins have a lower affinity towards MRP-mediated CMFDA efflux inhibition than that seen with MK571, a potent inhibitor of rodent Mrp- and human MRP-proteins (Leier et al., 2000). The findings in Chapter 2 indicated that simvastatin and atorvastatin inhibit uptake function of monocarboxylate uptake transporters to the same extent as that for phloretin, a typical inhibitor of the transporters. It could be argued that muscle toxicity caused by statins is attributed to their strong effect on the uptake transporters rather than efflux transporters or MRP particularly. Statins need to be transported first into muscle cells prior to the process of muscle fiber apoptosis (Copaja et al., 2011, Dirks and Jones, 2006) or statin-induced

mitochondrial impairment (Kwak et al., 2012, Golomb and Evans, 2008, Kaufmann et al., 2006). The efflux transporters in muscle cells, which are expressed on the sarcolemmal membrane of muscle fibers, were suggested by Knauer et al (2010) to rather have protective role against intracellular statin accumulation by their substrate affinity to the MRPs (i.e., MRP1, MRP4 and MRP5 particularly). However, as shown in this study, their affinity to MRP efflux inhibition were lower than that seen with the typical inhibitor of MRPs , MK571, suggesting lesser impact of statins on the efflux transporters compared to the their impact on MCT uptake transporters in the muscle. Although the findings in human muscle cells could be underestimated due to poor expression of the MRPs at both mRNA and functional levels, it is assumed that the hypothesis remains to be true as the same observation (i.e. low affinity inhibition to MRPs) was seen in high-MRP expression cell lines; HK-2 and L6 cells (see **figure 3.7** and **figure 3.13**).

As far as efflux transporters are concerned, MDR1 (ABCB1), MRP2 (ABCC2) and BCRP (ABCG2) remain to be important ABC transporters in statin disposition (Sissung et al., 2010). MDR1 and BCRP transporters have been regarded as the most characterised polymorphic efflux transporters (Lepper et al., 2005) and both of them mediate a wide range of substrate drugs including statins (Xiao et al., 2005, Gottesman and Ambudkar, 2001). Indeed, up to the present time, genetic polymorphisms of the abovementioned ABC transporters were also found to be associated with inter-individual variability in statin response and side-effects (Canestaro et al., 2014, Ferrari et al., 2014, Feng et al., 2012). Notably, the genetic mutations in the ABCG2 was reflected in statin bioavailability (i.e. atorvastatin and rosuvastatin) in general systemic exposure (Zhou et al., 2013, Keskitalo et al., 2009), but not at local exposure in skeletal muscle cells. Although the present study did not specifically elucidate the contribution of the three efflux transporters to statin-induced muscle toxicity, the findings are important as they provide a platform showing how modulation of transporter-mediated substrate dye efflux can be used as a high-throughput screening in cell cultures with high expression for the transporters. It can thus be suggested that the impact of statins on ABCG2 especially, can be assessed in the future either by using stably transfected HEK293 cells or high-ABCG2 expression cell line such as BeWo cells (Ceckova et al., 2006, Mueller et al., 2005). Among the three abovementioned ABC efflux transporters (i.e. MRP, MDR1 and BCRP), I found no evidence that statins inhibit MDR1-mediated H33342 dye efflux. Therefore, it was decided to only focus on the contribution of genetic polymorphisms of ABCC2 and ABCG2 genes that best represent efflux transporters in determining their association with

statin-related muscle symptoms in the patient population (further detailed in the chapter 4).

Chapter 4. Investigation of the Association between Genetic and Environmental Factors with Statin-Related Myotoxicity (SRM): A Case-Control Study

4.1 Introduction

Lipid lowering drugs, such as statins, have been demonstrated to significantly lower the incidence of cardiovascular events in at risk individuals. Whilst statins are generally well tolerated, statin-related myotoxicity (SRM) are relatively common (5 to 10%) and can rarely progress to severe and potentially fatal muscle toxicity, known as rhabdomyolysis. The factors contributing to the statin-related muscle toxicity remain largely unknown. However, there is evidence indicating that patient age, gender, concomitant therapy with certain drugs, increased drug exposure and genetic factors can promote SRM. There is also evidence that genetic factors, including those that influence statin disposition and localisation in skeletal muscle, contribute to SRM. The aim of the current study was to further elucidate the risk factors associated with SRM. Specifically, the study sought to determine the association between clinical, environmental and genetic factors (including polymorphisms in monocarboxylate transporters identified through my earlier cell-based work) and SRM**.**

4.2 Patients, Materials and Methods

4.2.1 Patients recruitments and blood collection

The study was approved by the Joint University of Newcastle and Health Authority Ethics Committee (**Appendix F**). The controls and cases, as defined below, were identified from a review of their medical notes prior to clinic attendance according to the inclusion and exclusion criteria described in **section 4.2.2**.

Controls

Patients who had received one of the two commonly prescribed statins (either simvastatin 40 mg nocte or atorvastatin 80 mg nocte) for at least 6 months without presenting with any muscle symptoms were recruited into the study. These individuals were recruited from the Lipid Clinic, RVI between May 2011 and January 2014.

Cases

Patients who had previously received either simvastatin or atorvastatin (or both) but had discontinued the drug because of muscle symptoms, thus clinically assigned as SRM cases, were recruited.

After obtaining informed written consent, patient demographic profiles, daily dose of statin used, indication for statin therapy, concurrent illness and medication, including those drugs known to affect the disposition of either simvastatin or atorvastatin were recorded using a structured questionnaire (**Appendix G**). Face-to-face interview about any muscle pain/weakness with the aid of a questionnaire or the review of medical records of those previously intolerant of simvastatin/atorvastatin was conducted by a trained research nurse.

A single blood sample (30 ml in total) was then collected from each patient. The blood sample was subjected to: (i) routine laboratory analyses (10 ml) for blood chemistries including liver function (alanine aminotransferase) and creatine kinase (CK) measurements (ii) centrifugation at 2500 rpm, on the same day (10 ml); the resultant plasma was stored at -20°C until further analysis. Another aliquot of whole blood (10 ml) was collected into EDTA tubes and stored without centrifugation under the same conditions prior to later DNA extraction for genotyping.

4.2.2 Criteria for SRM

The cases were defined as those presenting with a composite adverse events of any of the three outcomes as listed below, presenting within 6 months of starting therapy with a statin, consistent with the case identification criteria defined by Voora and co-workers (Voora et al., 2009) as follows:

i. Premature discontinuation of statins due to any side-effects;
ii. Myalgia/muscle cramps (irrespective of CK values) and clinically accessed as statin-related muscle toxicity by a clinician; and
iii. CK elevation >3-fold the normal range (ULN)

Cases corresponded to SRM2 to SRM5 according to the classification proposed by Alfirevic and co-workers (Alfirevic et al., 2014). To avoid vague, ambiguous, or

colloquial expression, all symptoms were recorded by staff in standard medical terminology in addition to the subject's own words. For example, symptoms were recorded as "myalgia," "muscle cramps," or "elevated createine phosphokinase" as appropriate. Additionally, patients' medical records were reviewed for symptoms of myalgia and their nature recorded.

The principle study inclusion criterion was having received either simvastatin or atorvastatin for at least 6 weeks. This was to ensure that patients had adequate exposure to either statin prior to study participation. The study exclusion criteria were; renal and/or insufficiency, hypothyroidism, diabetes mellitus, history of muscular complaints (either due to trauma or exercise), taking drug interacting with statin disposition, issues identified to affect compliance with treatment.

4.2.3 Single nucleotide polymorphism (SNP) selection

a. SNPs of known functional significance

The SNPs of known functional significance as listed in the **Table 4.1** were selected based on previous findings. Additionally, rs1050450 and rs713041 the SNPs in glutathione peroxidase 1 (GPX1) and GPX4, respectively, were selected on the basis of reduced GPX activity associated with these SNPs reported by several previous studies (Blein et al., 2014, Men et al., 2014, Hong et al., 2013, Monteiro et al., 2013, Larsen et al., 2013, Gautrey et al., 2011).

Table 4.1 SNPs selected for genotyping on the basis of previous genotyping studies on statin-related myotoxicity (SRM).

Gene	SNP	Evidence of the association with SRM/ reason for the selection	Reference(s)
SLCO1B1	rs4149056	OR for statin myopathy = 4.5, occurrence of SRM with p=0.03	Link et al., 2008, Voora et al., 2008
ABCC2	rs717620	Associated with switches to other cholesterol-lowering during simvastatin therapy	Becker et al., 2013
ABCG2	rs2231142	Markedly affect PKs of simvastatin acid	Tsamandouras et al., 2014, Keskitalo et al., 2009
CYP3A4	rs35599367 (*22 allele)	Associated with statin metabolism and response	Tsamandouras et al., 2014, Elens et al., 2013, Elens et al., 2011
COQ2	rs4693075	Associated with statin intolerance and myopathy	Puccetti et al., 2010, Marcoff and Thompson, 2007, Oh et al., 2007
GATM	rs9806699	Meta-analysis odds ratio for SRM = 0.60	Mangravite et al., 2013

Abbreviations: SLCO1B1, Solute carrier organic anion transporter family member 1B1; ABCC2, ATP-binding cassette, sub-family C member 2; ABCG2, ATP-binding cassette, sub-family G member 2; CYP3A4, Cytochrome P450 sub-family 3A member 4; COQ2, Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase; GATM, Glycine aminidotransferase; OR, odds ratio.

b. Selection of tagging SNPs in SLC16A1 and SLC16A3 genes

Since there was no prior information available on the influence of either SLC16A1 (MCT1) or SLC16A3 (MCT4) gene on statin-related myopathy, tag-SNP selection was employed for these two genes. For this approach, tag-SNPs in both MCT1 and MCT4 were picked from the HapMap website (http://www.hapmap.org). The tag-SNPs were generated after retrieving SNP genotype data for each gene such as frequency in population of interest and MAF cut-off value. For this study, data from CEU (European) population and a MAF cut-off value of 0.2 was used. The data resulted from tagging approach from the HapMap website captured 100 % of alleles with mean r^2 0.994 and 1.0, for SLC16A1 and SLC16A3 gene, respectively. The alleles captured (analysed on 12 December 2012) by the chosen SNPs in each gene are detailed in **Table 4.2**.

Table 4.2 Tag SNPs in SLC16A1 and SLC16A3 genes selected for genotyping.

GENE	TEST	ALLELES CAPTURED
SLC16A1 (MCT1)	rs9429505	rs7552903, rs4839272, rs2149036, rs4839270, **rs3849174**, rs7536532, rs9429505, rs7518984
	rs7556664	rs3789592, rs7169, **rs1049434**, rs6537765, rs7556664
	rs12028967	**rs4301628**, rs12028967
SLC16A3 (MCT4)	**rs7503429**	rs7503429
	rs12453290	rs12453290

Those SNPs (bold print) were chosen for genotyping in this study according to the evidence of MAF (>0.20) value obtained from 1958 British birth cohort website (http://www.b58cgene.sgul.ac.uk/).

4.2.4 Genomic DNA preparation and analysis

The DNA extraction was carried out by perchloric acid-chloroform extraction protocol as described by Daly and co-workers (Daly et al., 1998). In brief, 35 ml lysis buffer A [10 mM Tris [tris(hydroxymethyl)aminomethane] –HCl (pH 8.0), 320 mM sucrose, 5 mM magnesium chloride and 1% (v/v) Triton X-100] was added to 5 ml venous blood in a 50-ml polypropylene centrifuge tube. After mixing, the tube was centrifuged at 3,000 g (at 4°C) for 10 min. The supernatant was discarded and the cell pellet was re-suspended in 2 ml of solution B [(400 mM Tris–HCl (pH 8.0), 60 mM EDTA, 150 mM NaCl and 1% SDS]. A quantity of 500 µl of sodium perchlorate (5 M) was added and the sample was rotary mixed (Stuart Scientific) at room temperature for 10 min before incubating in a preheated hot block at 65 °C for 30 min for protein denaturation. Next, 2.5 ml of chloroform was added and the sample was mixed for 10 min at room temperature. The tube was then centrifuged at 1,400 g for 10 min, and the upper, clear DNA-containing phase was transferred to a new 15 ml polypropylene tube. Two volumes (5 ml) of cold 100% ethanol were added to the aqueous phase, and the tube was gently inverted until the DNA precipitated. The DNA was spooled using a soft plastic sterile loop and allowed to air dry for 5 min, then re-suspended by incubation in 200 µl of 5 mM Tris-HCl (pH 8) and left overnight at 50 °C (or at room temperature) in a sterile 1.5ml screw-cap micro centrifuge tube. Samples were quantitated and their quality assessed by absorbance measurements at 260 and 280 nm using NanoDrop 2000 (Thermo Scientific, UK). Specifically, this absorbance range is used to access the purity of DNA in which a 260/280 ratio of ~ 1.8 is generally accepted to indicate "pure" DNA. An absorbance unit of 1 at 260 nm is equivalent to 50 µg/ml double stranded DNA (dsDNA). The quantified DNA samples were then diluted to100 ng/µl as working stocks and were kept at 4°C. The remaining stocks, if any, were stored at -80 °C for long term storage.

4.2.5 Polymerase Chain reaction (PCR) used for genotyping by PCR-RFLP

Lyophilised oligonucleotide primers were resuspended from an initial concentration of 200 µM using sterile water and part of the stock was further diluted to 25 µM as working concentration. The working stock was stored at -4 °C while the remaining volume of stock concentration was stored at -20 °C. For each PCR reaction, genomic DNA (0.2 µg) was added and amplified in a total reaction volume of 20 µl containing; 0.1 mM dNTPs

(Bioline, London, UK), forward and reverse primers (both at 25 µM, Eurofins WMG, London, UK), 1x supplied buffer and 0.025 units Taq DNA polymerase (New England Biolabs, NEB, Hitchin, UK). The PCR amplifications were performed in 0.2 ml sterile microtubes (Fisher Scientific, Loughborough, UK), using an Applied Biosystem 2720 Thermal Cycler programmed to give the following cycling conditions; initial-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing conditions as indicated in **Table 4.3**, and extension at 72°C for 1 min and then eventually, final extension at 72°C for 7 min. The quality of PCR products were checked by electrophoresis on 2% agarose gels (see **Section 4.2.6a**).

The primer sets and the annealing temperature used for the PCR protocols were self-designed by our laboratory as indicated in **Table 4.3**. Their specificity was confirmed using a computer-based BLAST search from Genbank (http://www.ncbi.nih.gov). To digest SNP in rs1049434, the restriction enzymes (as well as their recognition site) were selected according to their ability to cut the recognition site (see **Figure 4.1**) from the NEBCutter V2.0 website (http://tools.neb.com/NEBcutter2/index.php) by inserting at least 10 bp of gene sequences around the targeted site for variant sequences of interest.

The restriction enzymes cut at their specific recognition site and digestion pattern between the wild-type and the variant sequences of interest were then differentiated according to the number of DNA bands produced. For example, since the mutation for rs717620 results in C to T substitution located in the 5'-untranslated region at position -24, TaqI enzyme which cleaves between T and C in the target sites 5'-TCGA-3' was selected for the digestion. The products resulted in digestion patterns which could be visualised on either agarose or acrylamide gel (see **Section 4.2.6**); the uncut DNA bands represented mutant individuals, the cut DNA bands represented wild-type individuals and heterozygotes containing both DNA bands.

Table 4.3 Primer set, PCR conditions and restriction enzymes used for PCR-RFLP assays.

Assay	Primer set	PCR conditions¶	PCR product (bp)	Restriction enzyme (Recognition site)
ABCC2 rs717620	Forward 5'-TGTCCATCCACTGTTTCAATG - 3' Reverse 5'-CTGGACTGCGTCTGGAT*C- 3'	Annealing at 54°C for 1 min	193	TaqI (TCGA)
ABCG2 rs2231142	Forward 5'- GTCTCATTAA AATGCTATTT - 3' Reverse 5'- CTCTTGAATG ACCCTGTTGA - 3'	Annealing at 50°C for 35 sec	151	MseI (TTAA)
SLC16A1 rs1049434	Forward 5'- TCAATGAACAACTGGTATGA- 3' Reverse 5'- ATGGAGACTACAAA TACACA-3'	Annealing at 55°C for 1 min	387	BccI (CCATC)

* T in primer sequence was replaced with A

¶Apart of annealing temperature described in Table 5.4, all PCR conditions were performed for 35 cycles, with denaturation at 94°C for 1min, extension at 72ºC for 1 min and was hold in the last cycle at 72ºC for 7 min to ensure elongation except for rs2231142. For rs2231142, the period for each step in each cycle was set for 35 sec instead of 1 min. All assays were self-designed by our laboratory. ABCC2, ATP-binding cassette, sub-family C member 2; ABCG2, ATP-binding cassette, sub-family G member 2; SLC16A1, Solute carrier family 16A; PCR, polymerase chain reactiom;bp, base pair; min, minute.

ABCC2

TTCAGTGGTT CCTTTTATGT ATGGCCACTC CTACAGAGGC CTCTTGTACT TTGGGAACTG
GTGAGTCTCC CTGTCCCTAG GGCTTTTTAG TCACATGTCCATCCACTGTTTCAATGTAAC
ATGCATCTAG GCAAGGTTAA YSRTTAAATG GTTGGGATGA AAGGTCATCC TTTACGGAGA
ACATCAGRAT GGTAGATAAT TCCTGTTCCA CTTTCTTTGA TGAAACAAGT AAAGAAGAAA
CAACACAATC ATAYTRATAG AAGAGTCTTCCTTCCAGACG CAGTCCAGGA ATCATGCTGG
AGAAGTTCTG CAACTYTACT TTTTGGGTGA GAAATTACTT TATCTTCATA TTGACTCTTC

SLC16A1

TTAACACAAC ACTCGAAATA GATGAATTCA GCAAAAATGG TTTAAGAAGT TAAGGCTCTC
TAGAGTTCTA AAGACTAAAA CTTAAGGCAC ATATTATAAT CTTTATGACA CTATTAAAAG
CTAAGATTAA AACAAAACAA AACAGAACCT ACTTCTTTCC CCCATCCTTT GCTACCAATC
ATTCCCTCCT CAAATTCAGG CTATTGGTAA GGAGTCAAAC AAAAATCCCA TCAATGAACA
ACTGGTATGATTTCCACACA AATGTCTACT ATTTGCATTG AGCACCACTG GTAGATTACA
GGCCAGTAGA ATATTTTCAG ATATCCTGGG TCATGAACTG CTCAATTTAC CCTTCAGCCC
CATGGATTCA GACTGGACTT TCCTCCTCCT TGGGCCCTCCWTCTGTGTCT TTCTGGTCCG
GAGATTCTGC TGCTTTGGTA ACTTCATTTG GCTTCCCAGC AACATCTATA CTGGTCTCTTC
CTCTTTACTT TCCTTTTTCT GCTCGTTTGC TTTCTGTTCT TTTGCCAAAA GTCGATAATT
GATGCCCATG CCAATGAAGA GATAGATACC TGAAATAATT AGGACGACGC CACATGCCCA
GTATGTGTATTTGTAGTCTCCATACATGTC

ABCG2

ATGGTCTTAG AAAAGACTCA TTATCATTAT GTCTCATTAA AATGCTATTT GCCTTAAGGA
TGATGTTGTG ATGGGCACTC TGACGGTGAG AGAAAACTTAMGTTCTCAGC AGCTCTTCGG
CTTGCAACAA CTATGACGAA TCATGAAAAA AACGAACGGATCAACAGGGT CATTCAAGAG
TTAGGTCTGG

Figure 4.1 FASTA sequences for ABCC2, SLC16A1 AND ABCG2 genes. The highlighted section (yellow) shows the position of both forward and reverse primers used for the PCR amplification and the red bolded character describes the studied SNP for the respective genes while the underlined sequences close to the SNP (red) are the recognition sites for the restriction enzymes. ABCC2, ATP-binding cassette, sub-family C member 2; ABCG2, ATP-binding cassette, sub-family G member 2; SLC16A1, Solute carrier family 16A.

4.2.6 Agarose and polyacrylamide gel electrophoresis (PAGE)

a. Agarose gel electrophoresis

The quality of PCR products and pattern of PCR products following digestion, were determined by agarose gel electrophoresis using 2% gels. The gels were made up by mixing agarose powder (Bioline, London, UK) with 1xTris-Acetate-EDTA (TAE) buffer i.e., 10-fold dilution from 1L of 10x TAE stock (48.4 g Tris), 11.4 ml glacial acetic acid [17.4M], 3.7 g [or 20 ml of 0.5M] EDTA, disodium salt), containing ethidium bromide (0.5 µg/ml). Prior to gel loading, 2 µl of 6x loading buffer (0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol, 30 % glycerol) was mixed with 4.5 µl of PCR product. Electrophoresis was carried out in 1xTAE buffer for 30-45 min at constant voltage of 100V and 50-60mA. The DNA bands were visualised with Fluro S-multi imager Quantity One (Bio-Rad Laboratories) and fragment sizes were estimated by comparison to a 100 bp DNA ladder (NEB).

b. Polyacrylamide gel electrophoresis (PAGE)

PAGE was normally used to visualise digested PCR products where better band resolution was needed due to small differences in band size. A 50 ml solution for a 10% gel was prepared by mixing 29 ml Milli-Q water, 5 ml 10xTAE stock (as described in **section 4.2.6a** above), 16 ml 30% amide-bisarylamide 29:1 (Fisher Scientific), 0.5 ml 10% (w/v) ammonium per sulphate (Bio-Rad, Hemel Hempstead, UK) and 100µl TEMED (Fisher Scientific). The gel was cast between two pieces of 200 mm x 200 mm glass plates separated by 0.8 mm spacer and allowed to polymerize at room temperature for about 30 min. Loading buffer (2 µl of 6x) was combined with 18 µl digested PCR products and applied to the gel. Electrophoresis was carried out at constant voltage of 180V for 2-3 h in 1x TAE buffer. The gel was stained with ethidium bromide (approximately 7 µl EtBr in 200 ml of 1xTAE buffer) for 10 min and fragment sizes were estimated by comparison to a 100 bp DNA ladder. Visualisation of the DNA fragments was then performed using Fluro S-multi imager Quantity One (Bio-Rad Laboratories) and fragment sizes were estimated by comparison to a 100 bp or 1000 bp DNA ladder (NEB), as necessary.

4.2.7 Genotyping assay

a. PCR-Restriction fragment length polymorphisms (PCR-RFLP)

Genotypes for polymorphisms in ABCC2 (rs717620), ABCG2 (rs2231142) and SLC16A1 (rs1049434) were determined by PCR-RFLP with the respective restriction enzymes listed in **Table 4.3**. PCR products were subjected to RFLP analysis by incubating the PCR product with the relevant enzyme (1.0 unit enzyme per µL of PCR product), as per manufacturer's instructions, at the following conditions; for TaqI, the amplification products were digested at 65°C for 5 hours while the MseI and BccI digestions were at 37°C for 24 and 5 hours, respectively. Bands of digested products were viewed either by 2% agarose (**section 4.2.6a**) or 10% polyacrylamide (**section 4.2.6b**) gel electrophoresis.

b. TaqMan SNP genotyping assay

Genotyping for SNPs listed in **Table 4.4 (**except for SNPs in GPX1 and GPX4) was carried out using TaqMan SNP genotyping assay. The assay was designed to detect variants of a single nucleic acid sequence, without quantifying the target. The presence of two probes in each reaction allows genotyping of the two possible variants at the SNP site in a target sequence. Each TaqMan® SNP genotyping assay consisted of a single, ready-to-use tube containing; i. 2 sequence-specific primers for amplifying the polymorphism of interest, ii. 2 allele-specific TaqMan® MGB probes for detecting the allles for specific SNP of interest. Each allele-specific TaqMan® MGB probe has two reporter dyes at its 5' end. Specifically, VIC® dye is linked to the 5' end of the Allele 1 probe while FAM™ dye is linked to the 5' end of the Allele 2 probe. For the context sequence of GAC,,,,[C/T],,,AAA of rs4301628 SNP for example, the VIC® dye-labelled probe binds to the G allele, and the FAM™ dye-labelled probe to the T allele. This means that Allele 1 VIC® dye-labelled probe corresponds to the first nucleotide inside the square brackets of the context sequence in the assay information file (AIF) shipped with each order. While, Allele 2 FAMTM dye-labelled probe corresponds to the second nucleotide inside the square brackets of the context sequence in the AIF. Therefore, three distinct genotype clusters (i.e., CC, CT and TT genotypes) from the intensity plot of an allelic discrimination assay using VIC® and FAM™ dyes were produced as shown in **Figure 4.2**. The reactions were prepared in duplicate by using 2x Taqman Universal Master Mix, 40x SNP Genotyping Assay Mix, DNase-free water, and 10ng genomic DNA in a final volume of 20µl per reaction. Positive controls and a no template control were included in

each assay as a quality control measure. The PCR amplification was performed using the ABI Prism 7000 Sequence Detection System machine (Paisley, UK) under the following thermocycler conditions: 10 min at 95ºC to activate the AmpliTaq Gold polymerase followed by 40 cycles of denaturation at 95 ºC for 15s and annealing/extension at 60 ºC for 1 min. The allelic discrimination results were determined using the SDS 2.2 software after the amplification by performing an end-point read.

Similarly, genotyping for SNP in GPX1 (rs1050450) and GPX4 (rs713041) was carried out by end point genotyping assay using TaqMan probes with assay ID of C_2561693_20 for rs713041, while a custom TaqMan assay was ordered for rs1050450 (assay ID:Hs00232019_CE, PCR/Sanger Sequencing Primer pairs were used). The genotyping assay was, however, performed using Lightcycler 480 Real-Time PCR System (Roche Applied Science, Burgess Hill, UK) under the following thermocycler conditions: 10 min at 95ºC to activate the AmpliTaq Gold polymerase followed by 50 cycles of denaturation at 92 °C for 15s and annealing/extension at 60 °C for 1 min, a cooling step was set at 40 °C for 10s. The genotyping for both rs1050450 and rs713041 were conducted by Jonathon Brown (Institute for Cell and Molecular Biosciences, Newcastle University).

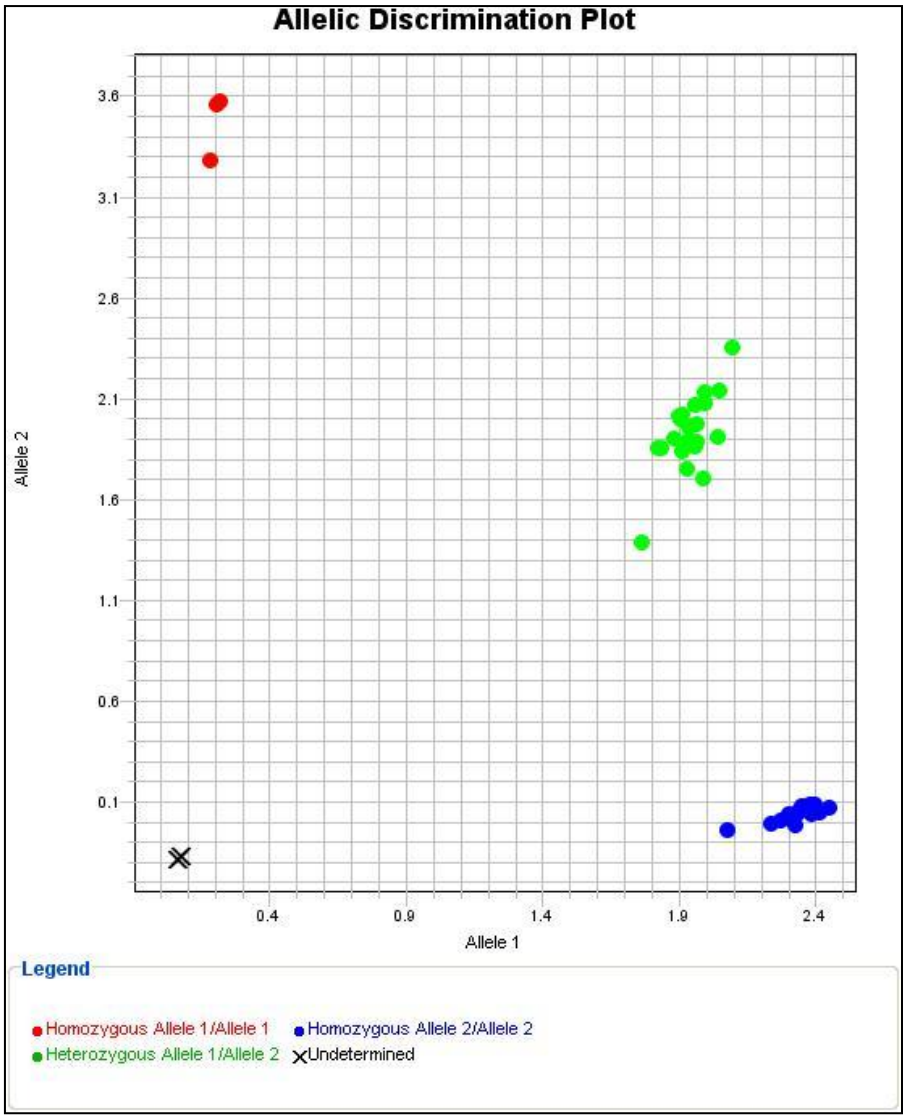


Figure 4.2 Samples results for the TaqMan SNP genotyping assay for rs4301628 in MCT1. Vertical axis represents relative fluorosence for FAM^{TM} label (marker of T- minor allele); horizontal axis represents relative fluorescence for VIC® label (marker of C- major allele). Cluster coloured blue is homozygous CC genotypes. Green coloured cluster is heterozygous CT genotypes while red cluster is homozygous TT genotypes. Cluster marked as X denotes the no template control (containing distilled water and TaqMan probes). SNP, single nucleotide polymorphism; MCT1, monocarboxylate transporter 1.

Table 4.4 SNPs genotyped using TaqMan SNP genotyping assay

Gene	SNP	Chromosome	Assay ID	Sequence (VIC®/FAM™)	MAF*
SLCO1B1	rs4149056	Chr.12: 21331549	C_30633906_10	TCTGGGTCATACATGTGGATATATG**[C/T]**GTTCATGGGTAATATGCTTCGTGGA	C=0.157
SLC16A1	rs3849174	Chr.1: 113460917	C_8696747_10	TCCTAATATGTAAAACGGGCACAGG**[G/T]**CACTGTTGTGAGGAAGAAAAGATTT	G=0.146
	rs4301628	Chr.1: 113492862	C_27970752_10	GACCCCAGGATAAAAATTTCTCATA**[C/T]**AGGAACAGAAGAGCAGGTCAATAAA	T=0.247
SLC16A3	rs7503429	Chr.17: 80191425	C_29221737_10	CTGAGGACCCTGGCGGGCTTCAAAC**[A/C]**CTGGGTCTGCCTGCGTCTCCCACCC	C=0.472
	rs12453290	Chr.17: 80190329	C-31557177_10	TGGGCGAGGTTTCTGGAGAGGTCCC**[A/G]**GAGGAGCGGGCTTGGGCAGCAGGGG	G=0.253
CYP3A4	rs35599367	Chr.7: 99366316	C_59013445_10	GTGCCAGTGATGCAGCTGGCCCTAC**[G/A]**CTGGGTGTGATGGAGACACTGAACT	A=0.090
COQ2	rs4693075	Chr.4: 84192168	C_28947992_10	TCTACCACAACTTTCCCACAAATCA**[C/G]**GCTCACATCAATTTCTTGAGTTGCT	G=0.427
GATM	rs9806699	Chr.15: 45740392	C_30104701_10	CCCCAGGCTGTCTGCTCCTGAGGGG**[A/G]**CTCTGGGGCATGGTGACATTCCCCA	A=0.298
GPx1	rs1050450	Chr 3:49357401	Hs00232019_CE	ATCGAAGCCCTGCTGTCTCAAGGGC[**A/G**]CAGCTGTGCCTAGGGCGCCCCTCCT	A=0.271
GPx4	rs713041	Chr.19: 1106615	C_2561693_20	CCGCCCGAGCCCCTGCCCACGCCCT**[C/T]**GGAGCCTTCCACCGGCACTCATGAC	T=0.478

*MAF values were obtained from 1000 genome browser website. Abbreviations: SLCO1B1, solute carrier organic anion transporter family member 1; SLC16A, solute carrier family 16A; CYP, cytochrome P450; COQ2, Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase; GATM, Glycine aminidotransferase; GPx, glutathione peroxidase.

4.2.8 Quality control of genotyping data

One disadvantage of a case-control study design compared with family-based association studies is the lack of an internal check on genotyping quality. Therefore, it is important to do a data-quality check to avoid any false-positive associations. In order to do that, genotype frequency was checked to be consistent with Hardy-Weinberg Equilibrium (HWE) for the control population, assuming the genotype frequencies in a population remain unchanged over the generations. The HWE can be defined using following formula (introduced by Godfrey H. Hardy and Wilhelm Weinberg in 1908);

$$\mathbf{p^2 + 2pq + q^2 = 1}$$

where, p is defined as the frequency of the dominant allele and q as the frequency of the recessive allele for a trait controlled by a pair of alleles (A and a). In other words, p equals all of the alleles in individuals who are homozygous dominant (AA) and half of the alleles in people who are heterozygous (Aa) for this trait in a population (Weir, 1996).

The HWE was assessed, according to the above formula, using a chi-square test and calculated using an online calculator at http://www.oege.org/software/hardy-weinberg.shtml. Any deviation from HWE could possibly be due to bias in selection, non-random mating (which is not expected here) or genotyping errors (e.g. Aa misclassified as AA or aa) during result interpretation which would cause the most concern. Any SNP that significantly deviated from HWE (i.e., at $P<0.05$) was not considered in further analysis.

In order to deal with inconsistency that may happen during reading PCR-RFLP results, randomly 10% of overall samples were blinded and randomly selected (~ 20%) and re-genotyped to confirm the authenticity of the results obtained earlier. Any missing data for genotyping (i.e failure to obtain blood samples from patient) resulted in exclusion from recruitment in the earlier stage of the study.

4.2.9 Statistical analysis

Statistical analyses for all clinical parameters (expressed as mean, 95 % confidence interval, CI) were performed using SPSS software version 21 (SPSS, Inc., Chicago, IL). In univariate statistics, chi-square statistics were used for categorical variables and t-tests for interval variables. In multivariate analysis, by taking a dichotomous variable (cases vs controls) as dependent variable, binary logistic regression was performed to assess independent clinical and genetic risk factor/s associated with the cases. Genotype frequencies was first determined to be in Hardy-Weinberg equilibrium (HWE) and compliance with the HWE (**section 4.2.8**) was determined for control groups to confirm that they met standard quality criteria using a web-based calculator available as described earlier. Univariate analysis of genotype frequencies between controls and cases were then compared using Fisher's Exact (two-tailed) test using an online calculator for 2x2 contingency table (http://vassarstats.net/odds2x2.html) where p-value, odds ratio, 95% confidence interval and chi-square for trend were calculated. A dominant model of genotypes [i.e. wild-type individuals vs (heterozygous + homozygous mutants)] was used for all SNPs studied due to homozygous mutants being rare. For all analysis, a p-value of <0.05 was taken as being statistically significant.

4.2.10 Sample size calculation

It is hypothesised that a multitude of factors contribute to the inter-individual variation in intolerance to simvastatin and atorvastatin in the general population, including clinical and environmental factors and common variants in the genes that mediate the drugs' pharmacokinetics and pharmacodynamics. However, as there is no *priori* information available on the variance in drug exposure (plasma simvastatin/atorvastatin concentration), and its impact on plasma CK concentration (an index of myotoxicity) and the quantitative effect of genetics and other confounders including patient characteristics on the risk of toxicity, a sample size of about 600 patients (500 controls and 100 cases) was deemed to be sufficient to test our hypothesis

4.3 Results

4.3.1 Clinical characteristics of study subjects

Patient recruitment was slower than initially anticipated. Consequently, the study target of 600 patients was not achieved by the end of my PhD studies. In effect a total of 430 hyperlipidaemic patients (314 controls and 116 cases) were recruited between May 2011 and April 2014. Subsequently all analyses were conducted based on 430 patients all of whom were European ancestry. The demographic and clinical characteristics of the cohort are summarised in the **Table 4.5**. There were slightly more female cases (58 %) than males (42 %). There was a greater proportion of patient on simvastatin therapy in both cases and control, with 84.5 % and 64.6 %, respectively, than atorvastatin and rosuvastatin ($P < 0.001$). In both groups, simvastatin 40 mg/day was the most frequently prescribed while the most common dosage regimen for atovastatin was 80 mg per day. There was no significant difference in age between cases and controls with the mean age being 59 years (95 % CI: 53-67) and 59 years (95 % CI: 50-67), respectively. Similarly, there was no significant difference in mean body mass index (BMI) between the cases (28.8, 95 % CI: 26.6-31.9) and the control group (28.7, 95 % CI: 25.9-32.9). The statin tolerant subjects (controls) had been on statin therapy longer than the cases (24 months, 95 % CI: 12-55 vs 8 months, 95 % CI: 3-18). There was no difference between the two groups for the commonly reported illnesses associated with statin-induced myopathy i.e.Type 2 Diabetes, hypertension and hypothyroidism. Similarly, there was no significant difference between the two groups for other types of medications use (**Table 4.5**).

Table 4.5 Demographic and clinical characteristics of patients who were statin-tolerant (control) and those who withdrew statin therapy due to perceived muscle symptoms (SRM cases).

Variables	**Control (n=314)**	**Cases (n=116)**	**P-value**
Sex			0.075*
Female, N (%)	151 (48)	67 (58)	
Male, N (%)	163 (52)	49 (42)	
Age, mean years (95% CI)	59 (50, 67)	59 (53, 67)	0.409
BMI, mean (95% CI)	28.7 (25.9, 32.9)	28.8 (26.6, 31.9)	0.822
Duration on statin (months)	24 (12, 55)	8 (3,18)	ND
Statin, N (%)			**0.000***
Simvastatin,	203 (64.6)	98 (84.5)	
10 mg/day	-	4 (4.8)	
20 mg/day	4 (2)	10 (12)	
40 mg/day	194 (95.6)	66 (79.5)	
80 mg/day	5 (2.5)	3 (3.6)	
Atorvastatin,	110 (35)	16 (13.8)	
10 mg/day	1 (0.9)	1(6.3)	
20 mg/day	2 (1.8)	-	
30 mg/day	1 (0.9)	-	
40 mg/day	11 (10)	-	
80 mg/day	95 (86.4)	15 (93.8)	
Rosuvastatin,	1 (0.3)	2 (1.7)	
Medical conditions, N (%)			0.544*
Type 2 Diabetes	27 (8.6)	8 (6.9)	
Hypertension	69 (22)	28 (24)	
Hypothyroidism	14 (4.5)	8 (6.9)	
None	65 (20.7)	20 (17.2)	
Co-medications, N (%)			0.523*
Non-hypolipidaemic medicines[#]	49 (15.6)	10 (8.6)	
Hypolidaemic medicines¶	23 (7.3)	3 (2.9)	

*Pearson Chi-Square test (2-tailed) was performed for the analysis
[#]The number presented was based on warfarin and omeprazole use, only one individual was on diltiazem from controls and cases, respectively.
¶All on fenofibrate. BMI, body mass index; ND, not determined.

4.3.2 Candidate Gene Association

The genotype frequencies of all studied SNPs were checked for their consistency with Hardy-Weinberg Equilibrium (**Section 4.2.8**). The genotype frequencies of all the SNPs, except for rs1050450 in GPX1for controls, fulfilled the Hardy-Weinberg equilibrium and were not significantly different to the frequencies reported in the GBR (British in England and Scotland) derived from 1000 genome browser (http://browser.1000genomes.org/index.html). All SNPs described in the following section, were successfully genotyped in 99.3 % of the patients (blood samples were missing for 3 individuals).

a. SLCO1B1

The SNP in SLCO1B1 gene is a non-synonymous rs4149056 (521T>C) SNP in SLCO1B1 at position 521 in the exon 5, that results in a Valine to Alanine substitution at codon 174 (Val174Ala). The results from Taqman genotyping produced a clear cluster separation for all samples genotyped similar to the example shown in **Figure 4.2**. This SNP was found to have borderline significant association with SRM by univariate analysis (P=0.059, OR=1.57, **Table 4.6**) and binary logistic regression (P= 0.047, OR = 1.59, **Table 4.7**), determined with other 11 SNPs studied as independent genetic factors.

Table 4.6 Association between individual SNPs and statin-related muscle toxicity by univariate analysis.

Gene and SNP	Genotype	Frequencies, n		P-value for HWE[a]	P-value[b]	OR (95% CI)
		Control[c]	Cases			
SLCO1B1 rs4145096	WT	224	72	0.293	**0.059**	1.57 (1.00-2.47)
	Het	77	42			
	Mut	10	2			
ABCC2 rs717620	WT	193	75	0.586	0.654	0.89 (0.57-1.39)
	Het	106	36			
	Mut	12	5			
ABCG2 rs2231142	WT	247	97	0.185	0.341	0.76 (0.43-1.33)
	Het	57	17			
	Mut	7	2			
CYP3A4 rs35599367 (*22)	WT	275	104	0.406	0.737	0.88 (0.44-1.76)
	Het	34	12			
	Mut	2	0			
COQ2 rs4693075	WT	123	48	0.165	0.740	0.93 (0.60-1.43)
	Het	154	52			
	Mut	34	16			
GATM rs9806699	WT	163	58	0.043	0.665	1.10 (0.72-1.69)
	Het	114	45			
	Mut	34	13			
GPx1 rs1050450	WT	168	58	<0.001	ND	ND
	Het	85	49			
	Mut	43	9			
GPx4 rs713041	WT	96	31	0.273	0.409	1.22 (0.76-1.97)
	Het	162	63			
	Mut	53	22			
SLC16A1 rs1049434	WT	91	36	0.28	0.812	0.92 (0.58-1.46)
	Het	163	54			
	Mut	57	26			
rs3849174	WT	199	65	0.326	0.146	1.39 (0.90-2.15)
	Het	96	47			
	Mut	16	4			
rs4301628	WT	137	60	0.84	0.190	0.73 (0.48-1.13)
	Het	140	47			
	Mut	34	9			

SLC16A3						
rs7503429	WT Het Mut	102 154 55	34 77 5	0.811	0.560	1.18 (0.74-1.87)
rs12453290	WT Het Mut	139 146 26	60 43 13	0.15	0.230	0.75 (0.49-1.16)

[a] P-value for HWE calculated in control group and compared with CEU population
[b] P-value derived from using Fisher's Exact test analysed between wild type individuals and combined heterozygous+homozygous mutant individuals in 2 x 2 tables.
[c] 3 missing values in control group
Abbreviations: HWE, Hardy-Weinberg equilibrium; SLCO1B1, Solute carrier organic anion transporter family member 1B1; ABCC2, ATP-binding cassette, sub-family C member 2; ABCG2, ATP-binding cassette, sub-family G member 2; CYP3A4, Cytochrome P450 sub-family 3A member 4; COQ2, Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase; GATM, Glycine aminidotransferase; SLC16A, solute carrier family 16A; GPx, glutathione peroxidase; WT, wild type individuals; Het, heterozygous mutants; Mut, homozygous mutants; OR, odds ratio; CI, confidence interval; ND, not determined.

Table 4.7 Association between selected SNPs and SRM according to binary logistic regression analysis.

Dependent variable	Controls	Cases	Explanatory variables	P-value[#]	OR	95% CI for Odds Ratio¶	
						Lower	Upper
Statin-related muscle toxicity	314*	116	SLCO1B1 (rs4149056)	**0.047**	1.59	1.01	2.52
			ABCC2 (rs717620)	0.639	0.90	0.57	1.42
			ABCG2(rs2231142)	0.247	0.71	0.40	1.26
			CYP3A4*22 (rs35599367)	0.656	0.85	0.42	1.73
			COQ2 (rs4693075)	0.866	0.96	0.62	1.50
			GATM (rs9806699)	0.842	1.05	0.67	1.62
			GPx4 (rs703041)	0.443	1.21	0.74	1.97
			SLC16A1 (rs1049434)	0.653	0.88	0.50	1.55
			SLC16A1 (rs3849174)	0.494	1.20	0.71	2.02
			SLC16A1(rs4301628)	0.318	0.77	0.45	1.29
			SLC16A3 (rs7503429)	0.750	1.09	0.66	1.79
			SLC16A3(rs12453290)	0.265	0.77	0.49	1.22

* Genotypes from 3 individuals were not included in the analysis.

[#] P-value for comparison of genotypes frequencies possession of one or two variant alleles between cases vs controls (reference) using multiple logistic regression; Hosmer and Lemeshow goodness-of-fit test was used as an indicator of the validity at the last step of iterations

¶ Odds ratio describes the odds to have at least one variant allele in the cases relative to the controls.

Abbreviations: HWE, Hardy-Weinberg equilibrium; SLCO1B1, Solute carrier organic anion transporter family member 1B1; ABCC2, ATP-binding cassette, sub-family C member 2; ABCG2, ATP-binding cassette, sub-family G member 2; CYP3A4, Cytochrome P450 sub-family 3A member 4; COQ2, Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase; GATM, Glycine aminidotransferase; SLC16A, solute carrier family 16A; GPx, glutathione peroxidase; OR, odds ratio; CI, confidence interval.

b. CYP3A4

The SNP in CYP3A4 gene evaluated in this study is rs35599367 which is usually referred to as CYP3A4*22 (http://www.cypalleles.ki.se/cyp3a4.htm). This SNP was genotyped by a TaqMan genotyping assay and the results produced a clear cluster separation for all samples genotyped resembling the example shown in **Figure 4.2**. There were no significant associations between genotypes and SRM by either univariate analysis **(Table 4.6**) or binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

c. COQ2

The SNP in COQ2 assessed in this study is rs4693075, encoding para-hydroxybenzoate-polyprenyltransferase, which participates in the biosynthesis of coenzyme Q10. This SNP was genotyped by TaqMan genotyping assay and the results produced a clear cluster separation for all samples genotyped resembling the example shown in **Figure 4.2**. There was no association between genotypes and SRM according to either univariate analysis (**Table 4.6**) or binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

d. GATM

The polymorphism, rs9806699, in glycine amidinotransferase (GATM), the gene encoding the enzyme regulating creatine biosynthesis was ascertained by a TaqMan genotyping assay and the results produced a clear cluster separation for all samples genotyped as shown in **Figure 4.2**. Since the p-value for lack of HWE in the controls showed only borderline significance (p=0.043), the SNP was still included in further statistical analysis. However, there was no association between genotypes and SRM according to either univariate analysis (**Table 4.6**) or binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

e. GPX1 and GPX4

Both the rs1050450 polymorphism in GPX1 and rs713041 in GPX4 were genotyped using TaqMan hybridisation probes. The p-value for SNP in GPX1 was <0.001, which was deviated from HWE so the SNP was excluded for further statistical analysis. The SNP genotyping assay for SNP in GPX4 produced a clear cluster separation for the three distinct genotype clusters (i.e., TT, TC and CC genotypes) but there was no association between

genotypes and SRM according to either univariate analysis (**Table 4.6**) or binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

f. ABCC2

The SNP in ABCC2 assessed in this study is rs717620 (C-24C>T) which is responsible for C to T substitution located in the 5'-untranslated region at position -24. The SNP was genotyped by PCR-RFLP (see **Figure 4.3** for a typical result using 2% agarose [a] and 10% acrylamide [b] gel). The SNP was successfully genotyped in 97% individuals in the study cohort. However, there was no association between genotypes and SRM according to either univariate analysis (**Table 4.6**) or binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

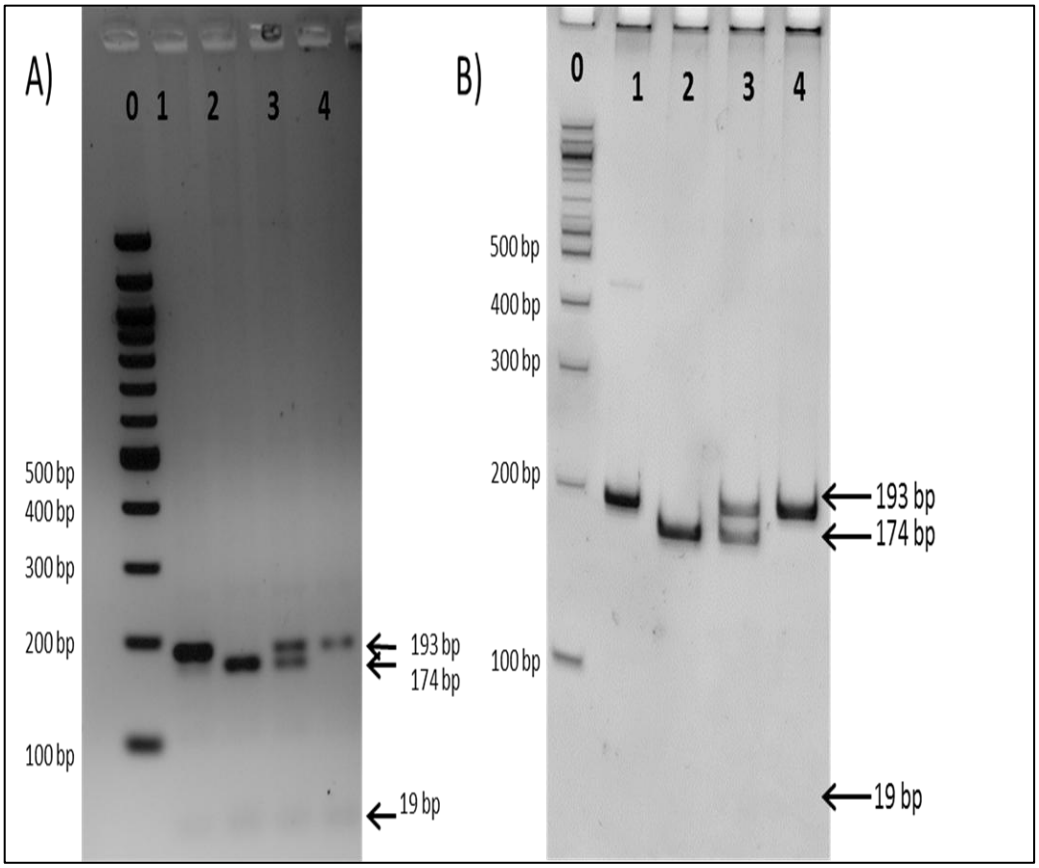


Figure 4.3 Genotyping of rs717620 (-24C>T) in ABCC2 by PCR-RFLP-based assay on (A) 2% agarose gel and (B) 10% polyacrylamide gel. Lane 0, 100 bp marker ladder; lane 1, uncut PCR product; lane 2, CC genotype (174 bp); lane 3, CT genotype (193-, 174- and 19 bp) and lane 4 (uncut), TT genotype (193 bp). The 19 bp fragment in (B) was invisible in the gel owing to its fast migration speed. ABCC; ATP-binding cassette, sub-family C member 2; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; bp, base pair.

g. ABCG2

The SNP rs2231142 in ABCG2 was examined by PCR-RFLP (see **Figure 4.4** for a typical result using 2% agarose gel). There was no association between genotypes and SRM according to either univariate analysis (**Table 4.6**) or binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

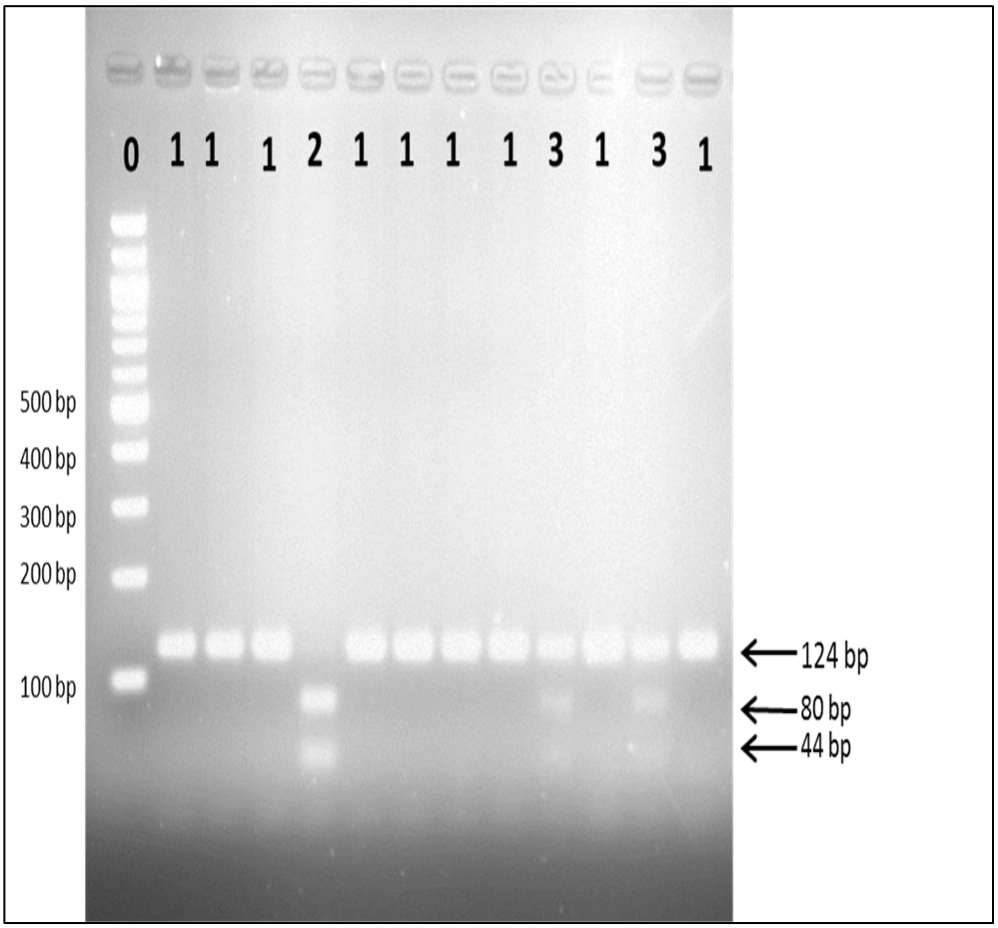


Figure 4.4 Genotyping of rs2231142 in ABCG2 by PCR-RFLP-based assay on 2% agarose gel. Lane 0, 100 bp marker ladder; lane 1 (uncut), CC genotype (124 bp); lane 3, CA genotype (124-, 80- and 44 bp) and lane 2, AA genotype (80- and 44 bp). ABCG2, ATP-binding cassette, sub-family G member 2; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; bp, base pair.

h. SLC16A1

Three tag-SNPs in SLC16A1 were selected for this study (i.e.,rs1049434, rs3849474 and rs4301628). Of the three SNPs, rs1049434 was analysed using PCR-RFLP (see **Figure 4.5**) while the other two were assessed using TaqMan genotyping assays. For rs3849474and rs4301628, the results produced a clear cluster separation for all samples analysed resembling the example shown **in Figure 4.2**. The SNPs were successfully analysed in 100 % the patients. However, there were no associations between the three tag-SNPs and SRM analysed by both univariate analysis (**Table 4.6**) and binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors

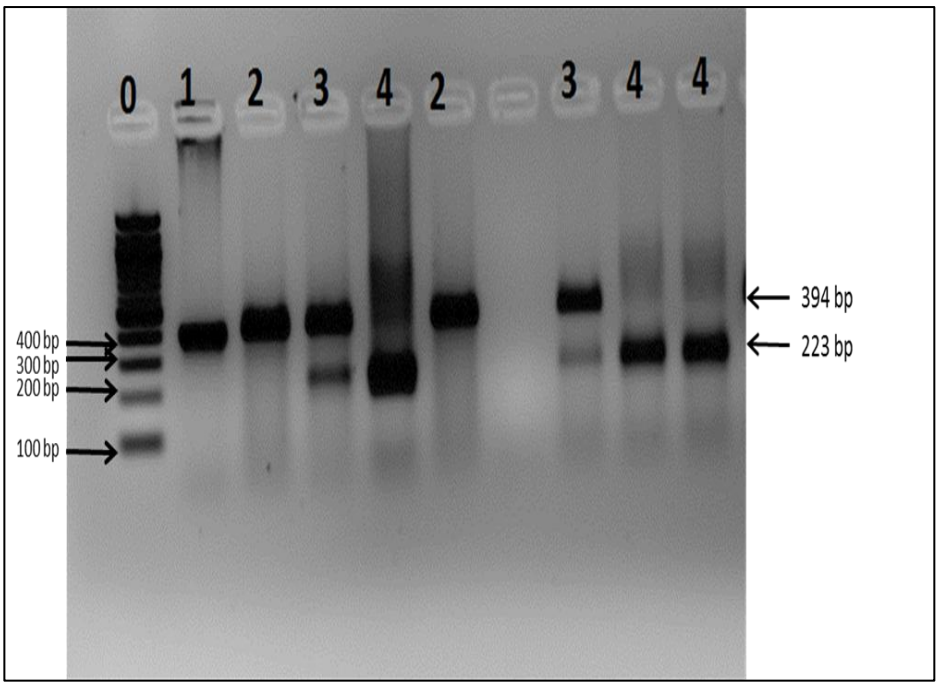


Figure 4.5 Genotyping of rs1049434 in SLC16A1 by PCR-RFLP-based assay on 2% agarose gel. Lane 0, 100 bp marker ladder; lane 1, uncut PCR product; lane 2 (uncut), TT genotype (394 bp); lanes 3, TA genotype (394-and 223bp) and lanes 4, AA genotype (223 bp). SLC16A1, solute carrier family 16A, member 1; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; bp, base pair.

i. SLC16A3

Two tag-SNPs in SLC16A3 were selected in this study (i.e., rs7503429 and rs12453290). Both were genotyped using TaqMan genotyping assays and the results produced a clear cluster separation for all samples genotyped resembling the example shown in **Figure 4.2**. The SNPs were successfully genotyped in 100% individuals in the study cohort. There were no associations between genotypes and SRM for either rs7503429 or rs12453290 in univariate analysis (**Table 4.6**) and binary logistic regression (**Table 4.7**), determined with other SNPs studied as independent genetic factors.

4.3.3 Multivariate analysis of data

The SLCO1B1 rs4149056 individuals were further stratified according to the type of statins (simvastatin and atorvastatin) used (**Table 4.8**). A borderline significant different in C allele frequency was detected between cases and controls (X^2 [1, N = 427] = 0.902, P = 0.059), regardless of the type of statin used. In the cases, the possession of C allele was not associated with the use of either simvastatin (X^2 [1, N = 286] = 0.902, P = 0.393) or atorvastatin (X^2 [1, N = 126] = 1.828, P = 0.239).

Table 4.8 Univariate analysis comparing the distribution of SLCO1B1 rs4149056 genotypes in both cases and controls and stratification according to the type of statin

Statin-type	Cases			Controls			C allele frequency		
	T/T	T/C	C/C	T/T	T/C	C/C	Cases	Control	P-value*
All	72	42	2	224	77	10	0.198	0.156	0.059
Simvastatin	55	26	2	146	49	8	0.18	0.16	0.393
Atorvastatin	9	7	0	80	28	2	0.219	0.145	0.239

* P-value derived from Pearson correlation using Fisher Exact test using a dominant model of genotypes

Further multivariate analysis for the SLCO1B1 rs4149056 genotypes controlled for the effect of other patient factors such as age, BMI and gender revealed that rs4149056 (dominant model) had a borderline significant association with SRM (**Table 4.9**) between case and control (P = 0.073; odds ratio = 1.52, 95 % CI = 0.96-2.39). Patient age and BMI made no significant contribution to the regression model. Interestingly, patient gender, after controlling for other factors indicated in the **Table 4.9**, also exhibited a borderline significant association with SRM (P=0.057; odds ratio = 1.53, 95 % CI = 0.99-2.37) between cases and controls with odds ratio of 1.53.

Table 4.9 Multivariate analysis using logistic regression for cases vs. control to control for the effect of factors which are relevant to SRM

Variables	OR (95% CI)	P-value*
SLCO1B1 rs4149056 (Reference: TT genotypes)	1.52 (0.96 – 2.39)	0.073
Age	1.01 (0.99 – 1.03)	0.305
Sex (Reference: Male)	1.53 (0.99 – 2.37)	0.057
BMI	1.00 (0.96 – 1.04)	0.923

*Calculation assume dominant model for SLCO1B1 rs4149056 genotype. SLCO1B1, Solute carrier organic anion transporter family member 1B1; BMI, body mass index; OR, odds ratio; CI, confidence interval.

4.4 Discussion and conclusion

Here I describe the outcome of an interim analysis of an ongoing, population-based case-control study of the association between genetics, drug exposure and SRM symptoms. The current analysis was limited to a subset of 430 patients for which data were available (314 controls, 116 cases), instead of the planned number of 600 patients (500 controls, 100 cases) which is the final study recruitment target.

The data are consistent with the accumulating evidence on the role of single nucleotide polymorphism (SNP) in SLCO1B1 gene (c.521T>C, p.V174A; rs4149056), the gene that codes for the hepatic OATP1B1 uptake transporter, in determining susceptibility to SRM (Wilke et al., 2012, Voora et al., 2009, Link et al., 2008). The data show a borderline association (with P=0.047 in multivariate analysis, **Table 4.7**) of the SLCO1B1 rs41490565 variant allele with statin myopathy with an odds ratio of 1.57 for carriage of the variant. However, a Bonferroni correction for multiple testing based on the total number of genotyping tests resulted in absence of any significant signal. The SLCO1B1 result reported here therefore needs to be treated with caution. In previous studies including the STRENGTH (Statin Response Examined by Genetic Haplotype Markers) study in 2009, the association of the SLCO1B1 rs41490565 with adverse events in patients assigned to simvastatin appeared to be the strongest and associated with creatine kinase (CK) elevations (Voora et al., 2009), although this was not the case with other population such as Greeks (Giannakopoulou et al., 2014). Voora and colleagues also found that, when patients treated with various statins were considered, myalgia was not correlated with CK elevations (Voora et al., 2009). It seems that the occurrence of the muscle symptoms was linked to the presence of the SNP, rather than the CK levels directly, although CK level has previously been identified as a biomarker for muscle disorders. The present study was based on cases who reported muscle symptoms, regardless of CK levels (refer **section 4.2.2** for case identification).

Carriage of SLCO1B1 rs4149056, remained the strongest genetic predictor for SRM, albeit of borderline significance (p=0.047), after controlling for the 11 other SNPs that were studied. However, it should be pointed out that the results are based upon an inadequate sample size. Patient recruitment for the study is ongoing at the time this thesis was written. It is thought that the association between SLCO1B1 and SRM will become more obvious when the study is adequately powered. When the SLCO1B1 rs4149056 was corrected for

the effect of other patient factors such as age, sex and BMI, the effect of SLCO1B1 was lost. Epidemiological studies have shown that advanced age, female sex (linked to small body frame) and frailty, increase susceptibility to SRM (Armitage, 2007, Schech et al., 2007, Bays, 2006) thus supporting the contribution of patient gender to this clinical consequence. The contribution of rs4149056, gender and to a certain degree, BMI, for the susceptibility to SRM have been confirmed further in a post-hoc analysis of data on 600 patients, which was the originally calculated sample size for this study (refer **Appendix H)**.

The study findings did not support those reported by Brunham et al. (2012) who demonstrated that the differential effect of the SNP was stronger for simvastatin users compared with atorvastatin in the Netherlands patient population. There was a strong association documented between rs4149056 and the risk of statin-related myopathy among simvastatin users in the SEARCH (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine) trial (Link et al., 2008) and most recently by Carr and colleagues (Carr et al., 2013). However in the current study, I found no evidence of an association between the SNP and either simvastatin or atorvastatin. As shown in **Table 4.8**, after stratification of SLCOIB1*5 genotypes according to the type of statin that each case and control was prescribed, there was no difference in genotype frequency between cases and controls for either simvastatin or atorvastatin users. However, this may well be due to the study not being sufficiently powered as demonstrated with the small effect size. It is anticipated that an adequate sample size (with the same MAF value for the SLCO1B1 rs41490566 as presented in the current data) will increase the existing small effect size to a small-to-medium effect size at $p<0.05$. Uncertainties, however, remain for atorvastatin users, since some of them had been switched from simvastatin and were therefore not entirely representative for atorvastatin group and therefore resulted in higher effect size than that seen with simvastatin sub-group. It might be that the atorvastatin sub-group cases might be contaminated with those intolerant to simvastatin and thus, the question whether the lack of different association to the SRM cases between these two statins remains inconclusive. Furthermore, the contribution of SLCO1B1 to SRM may be less for atorvastatin transport than for simvastatin as demostrated by the plasma drug concentration versus time area under the curve (AUC) reported by several previous studies. Confirming this, C allele carrries for the SLCO1B1 rs4149056 had a significantly increased mean AUC of active simvastatin acid (3.2-fold), atorvastatin (2.4-fold), and rosuvastatin (1.7-fold) compared with c.521TT genotype, whereas no effect was seen for simvastatin lactone and fluvastatin (Pasanen et al., 2007, Niemi et al., 2006, Pasanen et al., 2006). A further study

with a prospective case-control design is warranted to elucidate the true impact of the SLCO1B1 rs4149056 among both simvastatin and atorvastatin users on the occurrence of SRM.

The present study shows no evidence of contribution of newly proposed candidate genes in SRM. It is interesting to note that carriers of rs9806699, a variant in GATM, a gene that encodes the rate-limiting enzyme in creatine production, highlighted as an emerging factor in pharmacogenetics of statins (Canestaro et al., 2014, Norata et al., 2014), was not associated with SRM in my study. The rs9806699 was first demonstrated by Mangravite et al. (2013) to contribute statin-related side effects, although this finding was not supported by later studies (Luzum et al., 2015, Carr et al., 2014, Floyd et al., 2014). Carr et al. (2014) showed that the minor allele frequency (MAF) of the SNP was not significantly different between controls (n = 587) and myopathy cases (n = 150) with odds ratio of 0.94 (P = 0.68) and 'severe' myopathy (creatine kinase, CK, > x10ULN) cases (n=37) with odds ratio of 0.94 (P = 0.83). Additionally, Luzum et al. (2015) failed to replicate the protective effect of the GATM reported by Mangravite et al. (2013), using case-control analyses of statin myopathy in 715 Caucasians. Despite it was once claimed promise as a candidate for statin-related myopathy, it is not known and remains controversial (Ballard and Thompson, 2013) as to why the rs9806699 carrier status was associated with reduced incidence of statin-related myotoxicity (Mangravite et al., 2013). Consistent with the subsequent findings by Carr and colleagues (2013), I found no association between rs4693075 in COQ2 gene and SRM. Similarly, CYP3A4*22 was not associated with SRM cases even though the SNP rs35599367 is associated with reduced CYP3A4 activity which is relevant to the metabolism of both simvastatin and atorvastatin, and has been found to be relevant to variability in simvastatin response in the Dutch population (Elens et al., 2011). Although proposed as a new promising SNP in personalised medicine (Elens et al., 2013), none of previous studies have found an association between rs35599367 and SRM. Finally, none of tag-SNPs from both SLC16A1 (MCT1) and SLC16A3 (MCT4), demonstrated an association with SRM. In particular, I found no association between SRM and the genes that determine statin exposure in muscle. Above all the genetic predictors studied, it seems that the best predictor of SRM is the SLCO1B1 rs4149056 which determines statin plasma concentrations.

In addition to genetic factor, other patient-specific factors such as gender also predict the susceptibility to SRM. In fact, patient-related risk factors have been reported to constitute a

major component for statin myotoxicicty (Taha et al., 2014). Despite the strong signal shown by SLCO1B1 genotype in a GWAS in 2008, efforts to determine other genetic contributory factors in SRM remain to be confirmed (Tsamandouras et al., 2014, Mangravite et al., 2013). Other genes may still contribute to SRM with lower effect sizes or via gene-gene or gene-environmental interactions but assessing such effects would require extremely large numbers of cases far in excess of those studied in the current study. However, the data from the current study suggest that patient gender, in addition to SLCO1B1 rs4149056 genotype, contributes to SRM susceptibility with a borderline significant effect. It is anticipated that a more definite association (with lower p-value) for SLCO1B1 and age will be detected once the final study target of 600 individuals is reached. Considering other parameters, the distribution of concurrent illness (Type 2 diabetes, hypothyroidism and hypertension) and polypharmacy (hypolipidaemic vs non-hypolipidaemic medicines) in general was not significantly different between cases and controls. This is important as lack of matching for these factors might have influenced the genetics findings. From large scale meta-analyses of statin trials, there is a suggestion for a diabetogenic effect of statins (Sattar and Taskinen, 2012). Furthermore, evidence which suggests an increased risk of diabetes was recently reported in patients treated with statins (van de Woestijne et al., 2014, Zaharan et al., 2013) but there was no suggestion that this occurred in the current study.

Until more recent times, there was no universally accepted method for assessing causality for statin-related adverse events (Agbabiaka et al., 2008). However, lately Alfirevic and colleagues have developed an algorithm that will help to assign phenotypes to SRM cases, based on clinical and biochemical parameters, including CK levels (Alfirevic et al., 2014). The SRM classification, adapted from the recommendation of the American College of Cardiology/American Heart Association/National Heart, Lung and Blood Institute Clinical Advisory Board (Pasternak et al., 2002), was developed by the group to standardize the degree of severity of SRM based on a defined algorithm i.e. SRM0 to SRM6, which described the clinical manifestation with and without CK elevation and/or evidence based upon muscle biopsy findings (**section 1.3.1**). Some hold the view that any assessment of the effect-size of genetics regarding SRM should be based upon measurement of the degree of CK elevation (Carr et al., 2013, O'Meara et al., 2014). Apart from CK, measurement of alanine aminotransferase, urine myoglobin levels (when clinically indicated), and renal function may be useful. In patients with a suspected autoimmune myopathy, the measurement of anti-HMGCR antibodies and muscle biopsy should be considered

(Alfirevic et al., 2014). Since CK levels were unavailable (at the time the case group had stopped statin therapy or during muscle pain had been reported), any conclusion on a possible correlation between CK levels and SRM cannot be made. Although most researchers have opted to use CK value (a non-specific marker for muscle damage) as a criterion to identify statin-related muscle toxicity (Link et al., 2008, Thompson et al., 2006, Ballantyne et al., 2003, Pasternak et al., 2002), its use alone remains controversial. In this study, statin intolerant cases were selected based on their clinical phenotypes, irrespective of CK levels. Although the CK levels were not a central focus for the inclusion criteria, majority of cases, but not all, were selected from SRM2 (muscle symptoms accompanied with CK <4xULN) cases with a few from SRM3, SRM4 and SRM5, (according to the recent classification by Alfirevic et al., 2014). After statin withdrawal, the reported muscle symptoms were then resolved (partially to complete disappearance) within 2 months.

In conclusion, this interim analysis showed an association between SLCO1B1 and SRM among statin (simvastatin and atorvastatin) users. However, it was not possible to show such association for the individual statins.

Chapter 5. General Discussion

In this chapter, I will be discussing the main findings of my research; (a) the impact of statins on the functional expression of membrane transporters studied at cellular levels (Chapter 2 and Chapter 3) and (b) from gene candidate study (Chapter 4) in the case-control study of statin-related myotoxicity (SRM). In particular, I will be focusing on implications of the findings and future research direction.

(a) Through my cellular work I showed that simvastatin and atorvastatin have a significant impact on monocarboxylate transporter (MCT), presumably MCT1, which is in keeping with previous findings (Ansari, 2007). This provided information that statins caused muscle toxicity by having a direct effect on muscle cells (thus affecting intracellular events including mitochondrial function). MCT1 is found to be present in almost all tissues, in many cases with specific locations within each tissue (Halestrap and Price, 1999). MCT1 is also localized in sarcolemmal membrane of mitochondrion (Brooks, 2009, Hashimoto and Brooks, 2008, Brooks et al., 1999), which is linked to SCL16A1 (MCT1 transcribing gene) polymorphism; it is possible that statins cause muscle toxicity by interfering with mitochondrial function. Indeed this has been demonstrated by both *in vitro* and *in vivo* regarding the mode of action of simvastatin (Bonifacio et al., 2014, Galtier et al., 2012, Kaufmann et al., 2006), suggesting that lipophilic statins are better at targeting MCT1 to induce mitochondrial toxicity than hydrophilic statins. Further work is thus needed to determine whether altered function of MCT1 either by inhibition with an inhibitor or by genetic mutation would exacerbate statin myotoxicity and/or mitochondrial toxicity.

According to the case-control study results (discussed below), I found no association between SLC16A1 polymorphism and SRM. Therefore, at this point, although it is conclusive that lipophilic statins (such as simvastatin and atorvastatin) modulate MCT1 function at cellular level, further studies are needed to assess whether reduced MCT1 function would have significant clinical consequences. It is worth exploring in the future the association between the polymorphisms in SLC16A1 or SLC16A3 (or its tag-SNPs) and clinical endpoints such as blood lactate level and/or any evidence of mitochondrial pathology. Last but not least, a site-directed mutagenesis could be adopted in order to access whether the polymorphisms in MCT1 result in differential drug transport *in vitro* followed by *in vivo* studies using animal models (e.g. gene knock-down).

In contrast to the effect on MCTs, lipophilic statins did not inhibit cellular MRP or MDR1 efflux transporters (Chapter 3). Consistently, none of genetic variant candidates of the ABC transporters (i.e., rs717620 and rs2231142, SNP in ABCC2 and ABCG2, respectively) resulted in any significance association with SRM among both simvastatin and atorvastatin users in the case-control study. Although both SNPs were considered to be critical determinants of both expression and activity of the efflux transporters and subsequently response to some drugs including statins (Bailey et al., 2010, Keskitalo et al., 2009, Han et al., 2007, Sparreboom et al., 2004), neither of them have yielded reproducible outcomes. In fact, very recently, the SNPs in the genes for the ABC transporters are considered to be less suitable genetic markers to predict therapeutic outcome of many clinically relevant drugs including statins (Bruhn and Cascorbi, 2014). It is unknown how statins impact BCRP function (as efflux transporter) at cellular level, which are highly expressed in the gastrointestinal tract and canaliculi, and are considered among the principal transporters involved in the bioavailability and systemic clearance of the majority of statins (Generaux et al., 2011). It is expected that both simvastatin and atorvastatin would have a low impact on BCRP efflux transporter, as there is no priori evidence of the involvement of the BCRP with lipophilic statins, except with rosuvastatin (Jemnitz et al., 2010, Kitamura et al., 2008) and pitavastatin (Fujino et al., 2005, Hirano et al., 2005).

Like all potential prognostic markers, the effect of polymorphisms on clinical endpoints must be validated through preclinical and clinical investigations. At pre-clinical stage, evidence for SRM at cellular level is supported by a number of ways as described by Taha et al. (2014). At clinical level, however, it is much more difficult to assess statin exposure at skeletal muscle level because it would require invasive procedures such as muscle biopsy. Assessment of biomarker such as creatine kinase (CK) levels is one way of predicting the degree of SRM (Ferrari et al., 2014, Carr et al., 2013), though a systematic review of 35 randomized, placebo-controlled trials of the six FDA-approved statins failed to show significant differences in CK elevations in statin-treated patients compared with placebo (Kashani et al., 2006). Furthermore, assessment of the CK level could be misleading as statin-induced myopathy has also been reported with normal CK levels in a case-report of four related patients from a Norwegian family (Troseid et al., 2005) suggesting that CK is not a direct product or biomarker for SRM. Interestingly in the case report, two of the four patients showed evidence of mitochondrial pathology with normal CK level, while the other two suffered muscle pain without any pathologic findings. It is likely that genetic factors, either directly or indirectly, affected mitochondrial pathology. It

is possible that in the former two patients defective lactate transport, as a result of a genetic defect of MCT1, had resulted in mitochondrial pathology with statins further suppressing the transporter function. In the cellular studies, the protocols adopted in the MCT uptake function studies to estimate cell numbers based on cell monolayer surface area became the major limitation. The protocols described in this thesis were based on relative comparison of the radiolabelled uptake to controls without treatment, and hence, explained huge variation in certain results. In future, it is suggested that the normalisation of the substrate uptake in cell monolayers be carried out using total cells number or protein measurement methods instead.

(b) The interim analysis of data relating to the case-control study confirmed what is already known about the association between SLCO1B1 polymorphism (rs4149056) and SRM (Carr et al., 2013, Brunham et al., 2012, Donnelly et al., 2011, Voora et al., 2009). The SNP in the SLCO1B1 gene, a predictor of toxicity for drugs such as methotrexate, erythromycin and docetaxel (Lima et al., 2014, de Graan et al., 2012, Lancaster et al., 2012), is likely to be considered as the first-level genetic predictor for SRM. Knowledge of SLCO1B1 genotypes is believed to have clinical utility for predicting SRM in patients on chronic statin therapy. SLCO1B1 polymorphism is found to be strongly associated with simvastatin-related myotoxicity according to a genome-wide association study, GWAS (Link et al., 2008). It has been suggested by Clinical Pharmacogenetics Implementation Consortium (CPIC) that SLCO1B1 could be used as a guide for effective management of patients receiving simvastatin therapy (Wilke et al., 2012). The SNP is still being regarded as the only convincing genetic predictor of SRM (Carr et al., 2013) which was also supported by my study findings.

The case-control study did not determine the association between SRM and statin type, dose and level of statin exposure. Therefore further work will be needed to evaluate such possible relationships. It seems that exposure (assessed by measurement of AUC) for all statins appears to be attributable to the SLCO1B1 polymorphism. It was found that, relative to the wild-type TT genotype, homozygosity for the C allele increases AUC by 221% for active simvastatin acid, 162%-191% for pitavastatin, 144% for atorvastatin, 57%-130% for pravastatin, 62%-117% for rosuvastatin and 19% for fluvastatin (Wilke et al., 2012). For simvastatin users especially, the possession of > C allele accounted for clinical sensitivity and specificity of 70.4% and 73.7%, respectively, for the prediction of definite or incipient myopathy during 5 years of 80 mg/day simvastatin use (Stewart, 2013). If the frequency of

C allele of the SNP was taken into consideration, the association of the SNP with statin response and side-effects may be true for certain populations such as Caribbean and Black African group in the UK with low MAF value for the SNP (Hippisley-Cox and Coupland, 2010), while it did not hold for other populations (Giannakopoulou et al., 2014, Lee et al., 2005). Among Caucasians, as this study suggests, it seems that rs4149056 attributes to the susceptibility of all grades of SRM, regardless of statin type (simvastatin and atorvastatin) and dose (40 mg or 80 mg).

A recent review by Mosshammer and colleagues (2014) implies that SRM is multifactorial and that other factors which directly influence statin exposure in striated muscle need to be taken into consideration. Also other than genetics, patient-related factors contribute to SRM (Taha et al., 2014). Despite the strong signal shown by SLCO1B1 genotype in an earlier GWAS in 2008 (Link et al., 2008), further efforts have been made to identify other genetic factors contributing to SRM (Tsamandouras et al., 2014, Mangravite et al., 2013). Gene candidate studies should be viewed in association with other genetic and non-genetic factors, through either gene-gene or gene-environmental association studies, in order to further elucidate the contributory factors to SRM. My study showed that SLCO1B1 rs4149056 genotype and gender contributed to SRM (although with borderline significance). However, I found no evidence for gene-gene interaction among the candidate genes studied. Although the variants in SLC16A1 and SLC16A3, (genes transcribing MCT1 and MCT4, respectively) resulted in significant alteration of MCT function at cellular level, polymorphisms in these genes were not significantly associated with SRM in our study patients classified as having mild-moderate muscles symptoms. Polymorphisms in these transportes could still play a role in the more severe SRM cases which might be worthy of further investigation.

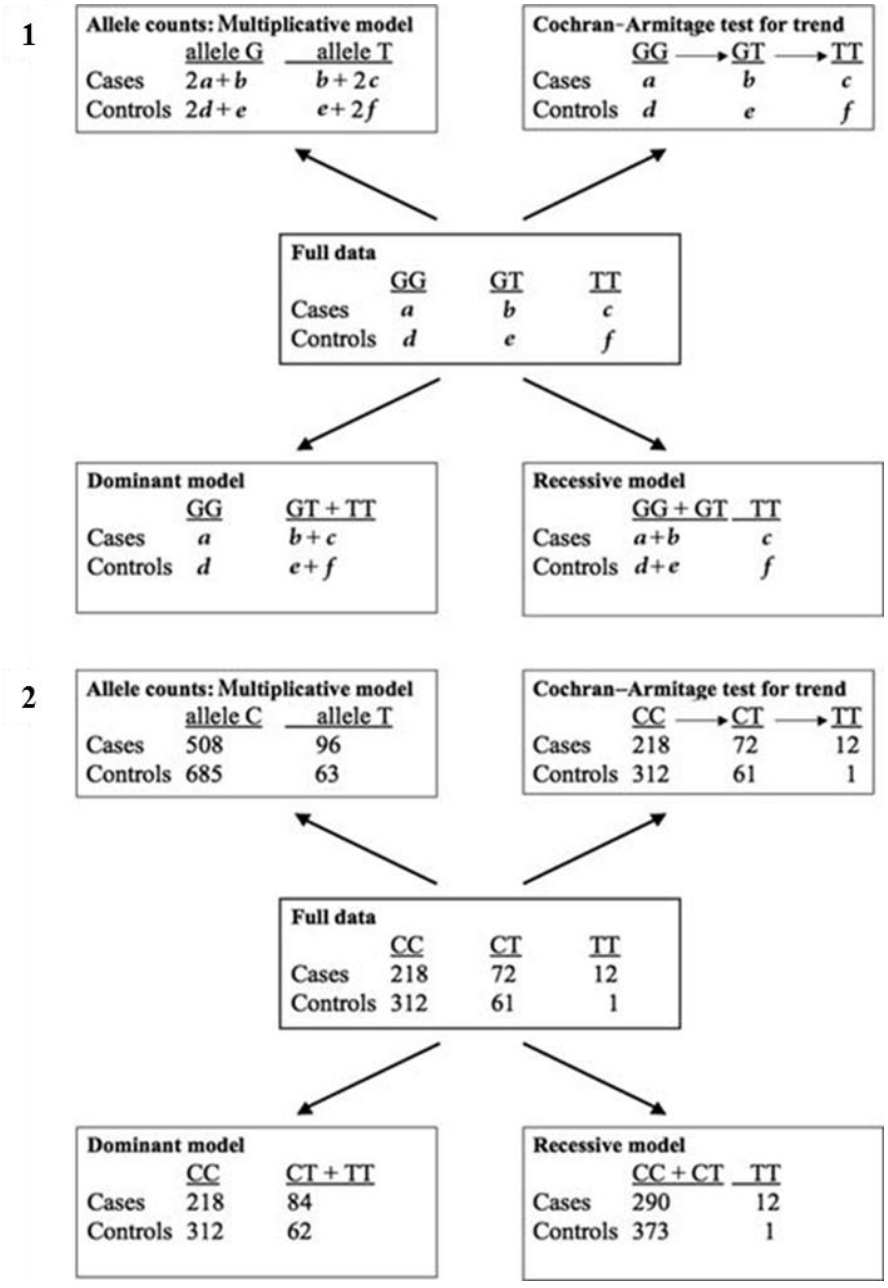
Overall, a small sample size was considered a limitation of the current case-control study. Because of slow recruitment the study did not reach its requisite target sample size of 600 patients by the time I had completed my PhD research. As such, I found only borderline association between SLCO1B1 polymorphism and patient factors of sex and BMI and SRM. However, a later post-hoc analysis of data based on 600 patients (refer to **Appendix H**), confirmed the presence of a strong association between SLCO1B1*5, patient sex, BMI and SRM as was previously reported in other patient populations (Hou et al., 2015, Joy and Hegele 2009).

According to previous pharmacogenetic studies, different threshold CK have been applied to classify statin-associated muscle intolerance (Carr et al., 2013, Brunham et al., 2012, Donnelly et al., 2011, Marciante et al., 2011, Voora et al., 2009, Link et al., 2008). For example, Donnelly and colleagues (2011) used CK levels within the range of 1-3x ULN as a composite definition of statin intolerance, whereas CK levels of more than 10x ULN has been used by others to classify subjects with SRM (Brunham et al., 2012, Marciante et al., 2011). The different classifications of SRM among these studies could have contributed to the differences in their findings. In the present study, however, the cases were predicted based on composite muscle adverse events, as described earlier (**section 4.2.2**), and not solely the CK levels. Despite the lack of CK levels the cases phenotypically corresponded to SRM2 to SRM5 according to the classification proposed by Alfirevic and co-workers (Alfirevic et al., 2014). For future work it will be important to assess the extent of contribution of SLCO1B1*5 to SRM in a more precisely defined patient group selected according to the standardised phenotype definitions (Alfirevic et al., 2014).

Last but not least, other than statins, there is also increasing focus of clinical studies on new LDL-lowering drugs for the treatment of hypercholesterolaemia. The new LDL-lowering drugs such as inhibitors to proprotein convertase subtilisin/kexin type 9 (PCSK9), apo B, and microsomal triglyceride transfer protein (MTTP) has been currently studied to ascertain their efficacy and safety. PCSK9 inhibitors such as alirocumab, evolocumab and bococizumab increase the hepatic uptake of LDL-C, while apoB and MTTP inhibitors decrease the synthesis and secretion of apoB-containing lipoproteins, for which they are all lead to marked reductions in plasma LDL cholesterol (Sahebkar and Watts, 2013). In term of efficacy, the PCSK9 inhibitors for example, are well tolerated and even patients who achieved very low LDL-C $\leq$ 0.65 mmol/l ($\sim$ 25 mg/dl) did not have any significant adverse effects (Reiner, 2015), however the outcome data with endpoints on their effects are still lacking and their long-term impact on cardiovascular events is currently under investigations (Sahebkar and Watts, 2013). Consistently with the apoB and MTTP inhibitors, a main safety concern i.e., the risk for hepatic steatosis, during treatment needs to be explored in prospective, long-term trials (Sahebkar and Watts, 2013). Above all those abovementioned drugs for the new lipid-lowering therapies, statins are remained to consider as the first option for lipid-lowering agents presumably due to lower cost. The favourable safety profile and cost-effectiveness of statin therapy is indeed undeniable (NCGC, 2014, Lazar et al., 2011).

Chapter 6. Appendices

APPENDIX A Different analysis methods used for SNP association studies for (1) arbitrary genotype counts and (2) for genotype counts in a case-control study. Adapted from Lewis and Knight (2012).



Briefly, Pearson's chi-square test can be used to assess departure from the null hypothesis in that case and controls have the same distribution of genotype counts (2-sided significance level). A higher frequency of a SNP allele or genotype in a series of individuals affected with a specific disease (or so cases) can be interpreted as the tested variant directly associates with the risk of the disease. In comparison with other genetic

markers such as microsatellite markers, insertion/deletions, variable-number tandem repeats (VNTRs), SNPs are the most widely tested markers in the genetic association studies.

In brief, the genetic variance can be divided into additive and non-additive genetic variance (Lynch and Wash 1998, Falconer and Mackay 1996). The non-additive parts include everything that has a nonlinear effect such as dominance (Wang et al., 1998), where the presence of just one allele contributes as much as two of the same allele, or epistasis (Lopez-Fanjul et al., 1999) where alleles act differently depending on what other alleles are present, or gene-environment effects where the contribution of an allele changes depending on the environment such as in the case of gene-dose effect of a drug. The fraction of the variance explained by the additive genetic effects, on the other hand, includes all the genetic effects. Since a single SNP has 2 alleles at each locus (termed as biallelic), the genotype counts for each group of the SNP between two groups (i.e., cases and controls) can be summarised in a different level of contingency table, as shown above. The first method known as multiplicative model in which contingency table may be decomposed from genotypes into alleles, with cell counts of the number of G alleles, and the number of T alleles carried by cases and controls, regardless of the genotype combination in which these alleles were carried (**Appendix A1**, upper left) and this test is valid under the null hypothesis of no association. When the true model of association is multiplicative (or log additive), the genotype relative risks for GG, GT, and TT genotypes can be modelled as 1, r, and r^2, with relative risk increasing by a factor r for each T allele carried (Sasieni, 1997). An alternative test for this model is the Cochran–Armitage test for trend (**Appendix A1**, upper right), which, as its name implies, tests for a trend in differences in case and control groups across the ordered genotypes in a 2x3 the contingency table. While other analysis methods may also be interpreted as an association based on the assumption that the disease risk may increase (or decrease) as the number of certain allele increases, assuming T (see dominant or recessive model) is a high-risk allele, in three genotype clusters i.e., CC, CT and TT. Additive model of inheritance may also be applied in a genetic association study and this model can be tested using Armitage test for trend as illustrated in the figure (**Appendix A1**, upper right). The model assumes an increased disease risk of r for GT genotypes, and 2r for TT genotypes. This model shows a clear trend of an increased number of GT and TT genotypes, with the risk for GT genotypes approximately half that for TT genotypes.

APPENDIX B Analysing qPCR data by the comparative $2^{-\Delta\Delta C}{}_T$ method (adapted from Schmittgen and Livak 2008).

1. **An example for the calculation of the fold change in expression level of a gene due to drug treatment**.

The mean C_T of the HOXD10 gene in treated and untreated samples was 24.6 and 27.5, respectively. The mean C_T of the 18S rRNA internal control in the treated and untreated samples was 9.9 and 9.8, respectively. What is the fold change in expression of the HOXD10 gene due to treatment?

Fold change due to treatment $= 2^{-\Delta\Delta CT}$

$= 2\text{-}[(24.6\text{-}9.9) – (27.5\text{-}9.8)$

$= 8$

2. **An example for the determination of the suitability of internal control for use in a drug treatment experiment.**

The gene expression is to be compared in cell cultures that are treated with a hypothetical drug to those that are untreated. The mean C_T from replicate runs of an internal control gene are 27.2, 27.0 and 27.4 (treated samples) and 26.2, 26.3 and 26.0 (untreated samples). What is the fold change in expression of the internal control in the treated versus the untreated samples? Does this gene serve as a useful internal control in this experiment?

Treated	**Untreated**
$2^{(-27.2)} = 6.49E\text{-}09$	$2^{(-26.2)} = 1.30E\text{-}08$
$2^{(-27.0)} = 7.45E\text{-}09$	$2^{(-26.3)} = 1.21E\text{-}08$
$2^{(-27.4)} = 5.65E\text{-}09$	$2^{(-26.0)} = 1.49E\text{-}08$
Mean = 6.53E-09	Mean = 1.33E-08

The fold change in the internal control in the treated samples compared to the untreated sample is: 6.53E-09/1.33E-08 = 0.490. Drug treatment reduced the expression of the internal control by 2.04-fold so it would not make a good internal control gene.

APPENDIX C An example for calculation of (A) ^{3}H-DL-lactate uptake and, (B) fractional ^{3}H-DL-lactate efflux rate in **section 2.2.6**.

1. Calculation of ^{3}H-DL-lactate uptake.

For example, uptake over 2 minutes of 50 μM [^{3}H]-DL-lactate at 1 μCi.ml-1 in 300 μl Krebs' (pH 5.5, Na^{+}-free) from a monolayer of 24-well plate was calculated as:

$$\text{DL-lactate uptake} = \frac{7546 \times 15{,}000 \text{ pmol}}{(6417 \times 30)} / 1.9$$

$$= 309.46 \text{ pmol.cm}^{-2}.(2\text{min})^{-1} \text{ or } 154.73 \text{ pmol.cm}^{-2}.(\text{min})^{-1}$$

2. Calculation of fractional ^{3}H-DL-lactate efflux rate

	Reading 1	**Reading 2**	**Reading 3**
Radioactivity collected on the 1st 2 min	4381	4032	3945
Radioactivity collected on the 2nd 2 min	1493	1390	1529
Radioactivity collected on the 3rd 2 min	567	587	506
Radioactivity collected on the 4th 2 min	348	316	366
Radioactivity collected on the 5th2 min	229	233	245
Radioactivity collected on the 6th 2 min	231	238	236
Radioactivity collected on the 7th 2 min	195	197	197
Radioactivity collected on the 8th 2 min	140	137	138
Final radioactivity remained in cells	2846	2821	2804
Total radioactivity	10430	9951	9966

Thus, fractional efflux rate (%) was gained as follow;

	Reading 1	**Reading 2**	**Reading 3**	**MEAN**
1	42.00384	40.51854	39.58459	**40.70232**
2	24.68177	23.4837	25.39445	**24.51997**
3	12.44513	12.96092	11.26447	**12.22351**
4	8.723991	8.016235	9.182137	**8.640788**
5	6.289481	6.425814	6.767956	**6.494417**
6	6.770223	7.014441	6.992593	**6.925752**
7	6.130148	6.244057	6.275884	**6.216696**
8	4.688547	4.631508	4.690687	**4.670247**

An example for the values in **Reading 1** from the table above were derived as follow;

42.00384 = 4381/10430*100

24.68177 = (1493/ (10430-4381)*100

12.44513 = (567/ (10430-(1493+4381))*100

8.723991 = (348/ (10430 – (4381+1493+567)*100

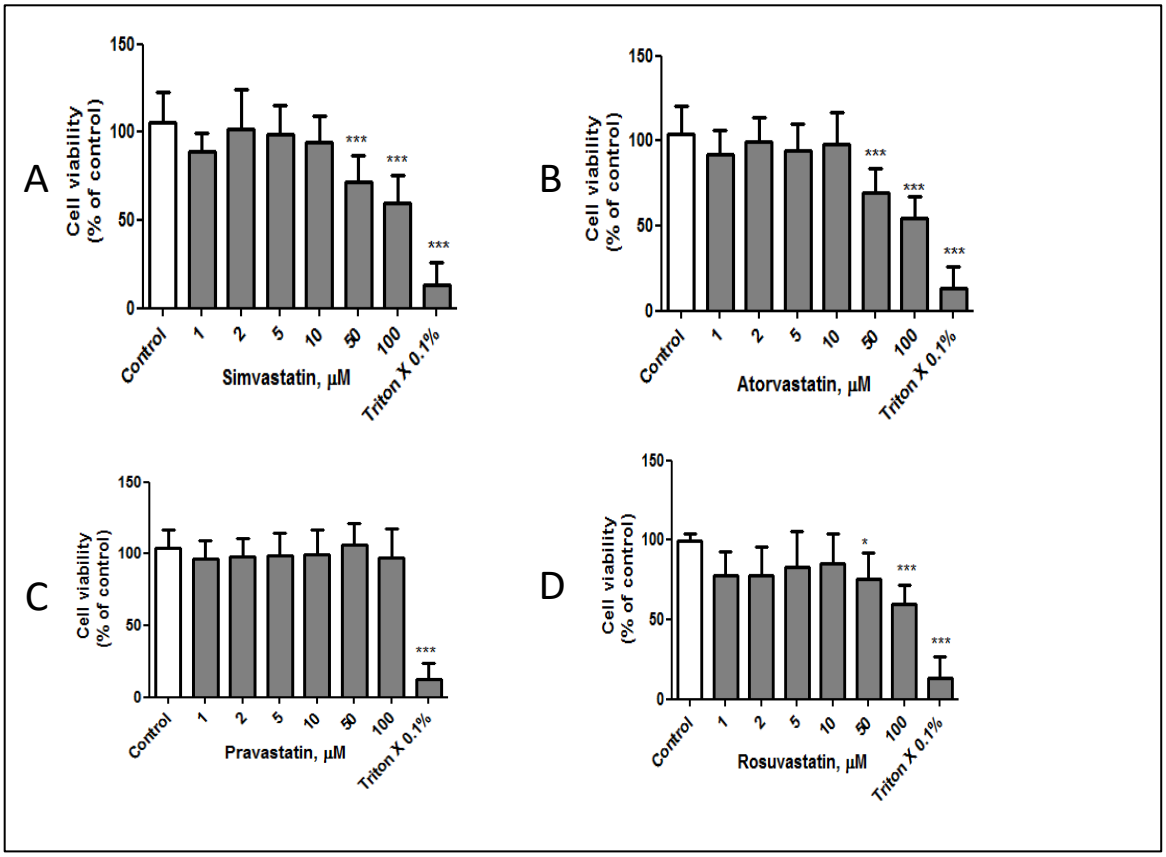
6.289481 = (229/ (10430 – (4381+1493+567+348))*100

6.770223 = (231/ (10430 – (4381+1493+567+348+229))*100

6.130148 = (195/ (10430 – (4381+1493+567+348+229+231))*100

4.688547 = (140/(10430 – (4381+1493+567+348+229+231+195))*100

APPENDIX D L6 cell viability for statin treatment for a range concentration of 1 µM to 100 µM



L6 myotubes were exposed to statins at a concentration range 1 to 100 µM for 48 hours, or to 0.1% triton-X-100 for 1 hour. For the determination of cell viability, L6 mytotubes were exposed to MTS reagents and incubated for 90 minutes at 37 ºC in the absence of light, after which absorbance of intracellular formazan was read at 490 nm.The background absorbance was substracted, and the readings were normalised to 100 % for cells exposed to solvent control and MTS reagent. Data are mean ± SD (n = 15), each n = 5 wells per concentration, from 3 independent experiments. Data are analysed by One-way ANOVA with Dunnett's post-test compared to solvent control; * P< 0.05, *** P < 0.0001.

APPENDIX E C_T values from RT^2 qPCR array results to determine drug transporters expression at mRNA level in L6 rat skeletal muscle cells in the presence and absence of simvastatin (2 µM) pre-treatment for 48 hours

Plate position	Gene	Gname	Test 1		Test 2		Test 3	
			Simvastatin-treated L6 cells	Control 1	Simvastatin-treated L6 cells	Control 2	Simvastatin-treated L6 cells	Control 3
A1	Abca1	-	30.76	29.03	28.83	26.2	30.75	28.48
A2	Abca13	-	33.32	31.7	31.43	27.54	31.97	29.77
A3	Abca17	-	28.77	31.85	28.94	27.75	29.32	29.65
A4	Abca2	Abc2	29.77	29.6	30.55	27.84	29.14	29.11
A5	Abca3	-	27.32	29.29	27.8	26.85	27.64	28.12
A6	Abca4	ABCR	31.65	31.67	31.59	27.77	32.57	30.48
A7	Abca9	-	27.3	26.09	28.52	26.91	26.69	26.99
A8	Abcb11	Bsep/Spgp	40	36.77	33.71	28.19	35.9	31.7
A9	Abcb1b	Abcb1/Mdr1/Pgy1	29.61	29.56	29.23	27.59	29.42	29
A10	Abcb4	Mdr2/Pgy3		32.91	32.47	28.59	37.44	31.61
A11	Abcb5	RGD1566342	40	31.74	31.79	28.02	33.92	29.89
A12	Abcb6	MGC93242	30.25	29.94	32.85	29.74	31.67	32.26
B1	Abcc1	Abcc1a/Avcc1a/Mrp/Mrp1	25.04	26.58	26.44	25.3	25.96	26.51
B2	Abcc10	-	35.22	33.03	34.77	30.66	34.51	33.12
B3	Abcc12	-	36.2	31.52	31.53	27.45	33.95	29.5
B4	Abcc2	Cmoat/Mrp2	31.9	30.76	30.86	26.86	32.06	29.01
B5	Abcc3	Mlp2/Mrp3	31.45	30.68	30.33	27.58	30.42	28.94
B6	Abcc4	Mrp4	30.63	30.65	31.71	29	30.07	30.97

B7	Abcc5	Abcc5a/MGC156604/Mrp5	28.67	29.3	28.9	27.43	28.93	29.49
B8	Abcc6	Mrp6	32.68	32.52	31.84	27.74	32.89	31.77
B9	Abcd1	RGD1562128	28.65	28.14	28.64	25.98	28.44	28.29
B10	Abcd3	PMP70/Pxmp1	26.69	26.58	26.94	26.98	26.46	27.08
B11	Abcd4	MGC105956/Pxmp1l	27.65	28.63	28.87	27.67	28.34	28.74
B12	Abcf1	Abc50	24.18	24.03	24.16	24.48	24.04	24.58
C1	Abcg2	BCRP1	36.25	30.69	31.14	27.14	32.29	28.67
C2	Abcg8	-	32	31.63	30.83	27.49	31.93	29.69
C3	Aqp1	CHIP28	30.53	33.14	29.93	27.99	31.52	31.62
C4	Aqp7	-	34.01	33.05	32.13	27.75	33.7	31.42
C5	Aqp9	MGC93419	40	33.23	32.25	28.01	32.63	31.5
C6	Atp6v0c	Atp6c/Atp6l	21.62	22.72	22.85	22.98	22.73	23.81
C7	Atp7a	Mnk	26.07	26.43	27.23	26.8	26.59	27.28
C8	Atp7b	Hts/PINA/Wd	34.47	31.55	32.3	27.74	32.24	29.97
C9	Mvp	-	25.87	25.83	26.67	26.16	26.1	26.86
C10	Slc10a1	Ntcp/Ntcp1/SBACT	32.58	31.89	31.24	27.77	31.68	29.72
C11	Slc10a2	ISBAT	35.62	30.95	31.22	27.63	32.07	28.81
C12	Slc15a1	Pept1		30.19	31.86	27.96	32.66	28.74
D1	Slc15a2	MGC91625	37.12	32.81	33.13	27.97	34.02	31.53
D2	Slc16a1	MCT1/RATMCT1/RNMCT1	24.97	25.12	25.58	25.28	25.57	26.42
D3	Slc16a2	-	26.76	28.18	27.82	27.09	27.63	28.58
D4	Slc16a3	Mct3	33.98	33.1	32.13	28.04	32.95	30.92
D5	Slc19a1	MGC93506/MTX1	28.07	28.19	28.92	28.2	28.91	29.66
D6	Slc19a2	MGC124887	27.95	29.12	28.46	26.86	28.62	28.85
D7	Slc19a3	-	35.79	34.09	32.61	28.2	32.66	31.29
D8	Slc22a1	MGC93570/Oct1/Orct1/Roct1	35.41	33.55	32.23	27.79	33.29	30.53
D9	Slc22a2	OCT2/OCT2r/rOCT2	35.49	31.59	31.3	27.31	32.72	29.6

D10	Slc22a3	-	40	32.78	32.27	27.72	33.62	30.8
D11	Slc22a6	MGC124962/Oat1/Orctl1/Paht/Roat1	37.19	34.69	33.09	29.24	35.66	33.17
D12	Slc22a7	Oat2	35.5	32.23	33.42	29.22	34.88	32.51
E1	Slc22a8	MGC93369/OCT3/Oat3/Roct	40	34.58	32.43	28.44	36.24	31.86
E2	Slc22a9	Oat5/Slc22a19	36.24	30.24	31.29	26.82	32.94	28.95
E3	Slc25a13	RGD1565889	30.13	29.47	30.05	27.76	30.63	30.19
E4	Slc28a1	Cnt1	37.4	31.44	31.85	27.94	34.34	30.25
E5	Slc28a2	-	30.54	31.54	30.29	26.59	31.86	29.17
E6	Slc28a3	Cnt3		31.6	31.57	27.52	33.61	29.56
E7	Slc29a1	rENT1	28.56	28.94	30.59	27.65	31.75	30.04
E8	Slc29a2	-	27.88	28.11	28.8	26.81	29.54	29
E9	Slc2a1	GLUTB/GTG1/Glut1/Gtg3/RATGTG1	33.46	30.29	34.58	32.79	34.92	35.28
E10	Slc2a2	GTT2/Glut2	27.56	31.6	27.02	26.08	27.79	27.97
E11	Slc2a3	GLUT3	27.87	28.18	30.59	27.58	29.73	30.04
E12	Slc31a1	Ctr1/LRRGT00200	26	26.92	26.99	26.55	25.72	26.71
F1	Slc38a2	Ata2/Atrc2/Sat2/Snat2	23.29	24.15	24.26	24.33	23.92	25.03
F2	Slc38a5	SN2	33.63	33.06	32.56	28.51	35.66	31.21
F3	Slc3a1	-	25.52	25.63	26.09	25.79	26.66	27.51
F4	Slc3a2	Mdu1	24.52	25.31	25.91	25.98	26.98	27.88
F5	Slc5a1	MGC93553/SGLT1	35.7	31.51	31.88	27.52	32.61	30
F6	Slc5a4a	Slc5a4	30.98	30.85	29.68	26.87	30.84	28.97
F7	Slc7a11	-	28.76	30.67	28.92	27.28	29.17	29.32
F8	Slc7a4	-	32.94	33.14	31.59	27.88	32.68	31.2
F9	Slc7a5	E16/TA1	25.51	27.74	27.99	27.58	27.15	28.56
F10	Slc7a6	-	28.47	29.02	28.95	27.16	28.77	28.65
F11	Slc7a7	y+LAT1	28.52	28.88	28.87	27.18	29.76	29.58
F12	Slc7a8	Lat2/Lat4	31.71	32.01	31.51	27.71	31.91	29.66

G1	Slc7a9	MGC114282	31.43	30.99	31.3	27.46	32.72	30.51
G2	Slco1a5	OATP-3/Oatp3/Slc21a7/Slco1a2	40	34.1	32.67	27.86	34.83	31.05
G3	Slco1a6	Oatp5/Slc21a13		32.23	32.57	27.67	33.09	29.8
G4	Slco1b3	OATP-4/Oatp4/Slc21a10/Slco1b2/rlst-1	37.69	32.87	32.78	27.87	33.77	30.69
G5	Slco2a1	Matr1/Slc21a2	32.83	29.89	30.59	26.18	32.51	28.81
G6	Slco2b1	Slc21a9/moat1	36.26	33	32.52	27.82	34.69	31.49
G7	Slco3a1	Slc21a11	26.15	26.59	25.77	25.49	26.07	26.65
G8	Slco4a1	OATP-E/Slc21a12	32.87	33.12	31.54	27.49	34.72	31.06
G9	Tap1	Abcb2/Cim/MGC124549	29.85	29.17	31.59	29.24	29.72	30.34
G10	Tap2	Abcb3/Cim/MGC108646	26.42	26.68	26.24	26.07	26.79	27.16
G11	Vdac1	-	22.71	22.63	23.81	23.32	23.49	24.16
G12	Vdac2	-	24.03	24.57	25.16	24.69	24.54	25.2
H1	Actb	Actx	18.57	19.5	18.45	18.84	19.67	20.23
H2	B2m	-	24	23.84	24.13	24.17	24.92	25.52
H3	Hprt1	Hgprtase/Hprt/MGC112554	21.01	21.21	20.82	21.17	21.75	22.54
H4	Ldha	Ldh1	21.5	21.89	23.05	22.65	22.71	23.44
H5	Rplp1	MGC72935	19.84	20.12	19.2	19.25	19.65	20.26
H6	RGDC	RGDC	37.66	33.09	34.1	33.55	30.11	32.82
H7	RTC	RTC	24.49	23.79	24.96	24.33	24.42	24.92
H8	RTC	RTC	24.48	23.83	24.91	24.26	24.43	24.87
H9	RTC	RTC	24.48	23.72	24.97	24.32	24.33	24.87
H10	PPC	PPC	19.15	20.02	19.42	19.72	19.45	19.66
H11	PPC	PPC	19.68	20.48	19.66	19.66	20.01	19.97
H12	PPC	PPC	19.51	20.31	19.6	19.52	19.7	19.91

APPENDIX F Ethical approval of the study

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

AT/JW

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30th November 2010

Professor F. Kamali
Professor of Human & Experimental Pharmacology
4th Floor William Leech Building
Institute of Cellular Medicine
Newcastle University

Dear Professor Kamali

Trust R&D Project:	**5448**
Title of Project:	**Investigation of the associations between genetics, drug exposure and statin-induced muscle toxicity**
Principal Investigator	**Professor Farhad Kamali**
Number of patients:	**550**
Funder (proposed):	**Malaysian Ministry of Health**
Sponsor (proposed):	**The Newcastle Upon Tyne Hospitals NHS Foundation Trust**
REC number:	**10/H0908/53**

Having carried out the necessary risk and site assessment for the above research project, Newcastle upon Tyne Hospitals NHS Foundation Trust grants NHS R&D approval for this research to take place at this Trust dependent upon:

(i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and understanding their responsibilities and duties (a copy of responsibilities prepared by the Trust R&D Office is enclosed)

(ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (*eg* CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (*eg* reporting of suspected adverse incidents).

(iii) where applicable, you, as Principal Investigator, should also adhere to the GMC supplementary guidance *Good practice in research* and *Consent to research* which sets out the good practice principles that doctors are expected to understand and follow if they are involved in research – see http://www.gmc-uk.org/guidance/ethical_guidance/5991.asp

Sponsorship

The Newcastle upon Tyne Hospitals NHS Foundation Trust will act as Sponsor for this project, under the Department of Health's guidelines for research in health and social care.

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In addition, the Trust has a Research Governance Implementation Plan, agreed with the Department of Health, in order to fully comply with Research Governance and fulfil the responsibility of a Sponsor.

As the Trust is acting as Sponsor for the research and where some of the research is taking place outside of Newcastle upon Tyne, then all costs must be met for research governance audit visits to those sites. It is the responsibility of the PI to provide confirmation to the Trust of who will pay these costs. Audit is required under the Research Governance Framework for Health and Social Care. (Please note that the Trust randomly audits 10% of approved research projects annually.)

Any changes to the study protocol, other study documents (eg, Patient Information Sheets and Consent forms), or any other amendments to the study must be submitted to the Ethics Committee and MHRA (if relevant) for review – see http://www.nres.npsa.nhs.uk/applications/after-ethical-review/amendments/ for guidance). The R&D office must also review these notices of amendments in parallel with ethical and regulatory review so that implications of the amendment can be assessed. Therefore, you must send a copy of all amendment documents to the R&D office at the same time you are submitting these to the Ethics Committee/MHRA. If changes or amendments to the study have implications for costs or use of resources, you must also submit details of these changes to the R&D office.

It is also the Principal Investigator's responsibility to ensure that all staff involved in the research have Honorary Research Contracts or the necessary letters of access. These need to be issued prior to commencing the research.

In addition, unless otherwise agreed with the Trust, the research will be covered for negligence under the CNST (Clinical Negligence Scheme for Trusts), however cover for no-fault harm is the responsibility of the Principal Investigator to arrange if required.

Please also note that for any NHS employee who generates Intellectual Property *in the normal course of their duties*, it is recognised that the Intellectual Property Rights remain with the employer and not the employee.

Yours sincerely

Amanda Tortice
Research Operations Manager

Enc: Principal Investigator Responsibilities Document

CC: Mr G Regan, Finance Department, Room 203, Cheviot Court, Freeman Hospital
Dr D Neely, Clinical Director, Royal Victoria Infirmary
Professor Kamali, Chief Investigator, Newcastle University
Ms A Hutchison, Research Nurse, Royal Victoria Infirmary

APPENDIX G Patient information sheets.

Investigation of the association between genetics, drug exposure and statin-induced muscle toxicity

Muscle Symptom Questionnaire (A)

(For patients currently on statin therapy)

Patient ID:_____

Hospital no:_____

Date of Birth:____/____/____

Sex: Male/Female

Today's Date: ___/____/____

Please try to answer the below questions as accurately as you can. Please feel free to ask if you are not sure of the answer to any of the questions.

Section A

1. Which statin are you currently taking?

Drug name ..... Daily dose (mg/day).....Duration.....(years).....(months)

2. For what reason are you taking this statin?

_____

3. Which medical conditions do you currently have? Please list them below:

_____

_____

_____

_____

_____

4. Are you taking other medications? ☐ Yes; ☐ No

If YES then please list them below:

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

5. Do you exercise in addition to your routine activity on a regular weekly basis (including walking, jogging, gardening)?

☐ Yes ☐ No

6. Do you have any muscle pain/weakness?

☐ Yes ☐ No

Please answer all the questions in Section B if your answer is YES to question 6. Otherwise please return the questionnaire to the researcher.

Section B

7. Is your pain triggered or aggravated by? (Tick those that apply)

☐ Physical exercise ☐ Rest ☐ Cold ☐ Lying in a certain way ☐ Fasting

8. Would you describe your pain as? (Tick those that apply)

☐ Cramps
☐ Stiffness
☐ Heaviness
☐ Muscular weakness or decrease of muscle power during physical activity (like trouble rising from a chair or walking up or down stairs)
☐ Tendonitis (ache, pain or local stiffness surrounding a joint in arm or leg)
☐ Other (please specify) _____
_____

9. How long have you had muscle symptoms? (Please tick only one box):

☐ 1–2 days ☐ <1 month ☐ 1–3months ☐ 3–12 months ☐ >12 months

10. Would you describe the pain/weakness you suffer from, as?

☐ Permanent (all the time), or ☐ Intermittent (for hours or minutes)

11. Did you have muscle pain/weakness before you started on statin therapy?

☐ Yes ☐ No ☐ Not sure

12. Have you had any form of surgery in the past month?

☐ Yes ☐ No ; If YES, what kind? _____

13. Is there any history of muscle disorders in your family or has any other member of your family has complained of muscle pain/weakness linked to taking statins?

☐ Yes; ☐ No

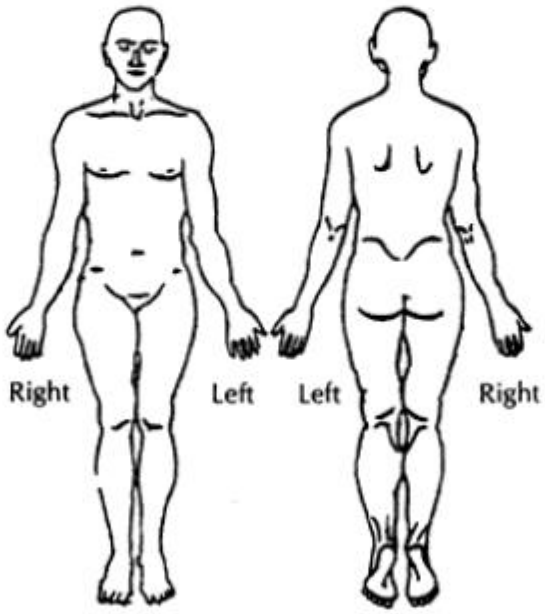
If YES then please give as much detail as you can below:

_____

14. Are you taking any painkillers for your muscle pain?

☐ Yes ☐ No

15. On the diagram below shade the areas where you have pain or other muscle symptoms. Put an X on the area which is affected most.



16. Please rate your pain by circling the number that best describes your pain on average

0	1	2	3	4	5	6	7	8	9	10
No pain										Pain as bad as you can imagine

17. Please circle the number that best describes the pain you have right now

0	1	2	3	4	5	6	7	8	9	10
No pain										Pain as bad as you can imagine

18. Check the appropriate answer for each item.

I believe my pain (or muscle symptom) is due to:

a. The effect of treatment (for example, medication, radiation, surgery, prosthetic device)

☐ Yes ☐ No

b. My primary disease (meaning the disease currently being treated and evaluated

☐ Yes ☐ No

c. A medical condition unrelated to my primary disease (for example arthritis)

☐ Yes ☐ No Please describe if answer is YES:_____

_____

19. Circle the one number that describes how, during the past week, pain (or muscle symptom) has interfered with your:

A. General activity

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

B. Mood

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

C. Walking ability

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

D. Normal work (includes work both outside the house and housework)

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

E. Relations with other people

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

F. Sleep

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

G. Enjoyment of life

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

Investigation of the association between genetics, drug exposure and statin-induced muscle toxicity

Muscle Symptom Questionnaire (B)

(For patients withdrawn from statin therapy because of muscle symptoms)

Patient ID:_____

Hospital no:_____

Date of Birth:____/____/____

Sex: Male/Female

Today's Date: ___/____/____

Please try to answer the below questions as accurately as you can. Please feel free to ask if you are not sure of the answer to any of the questions.

1. Which statin were you previously taking?

Drug name ..... Daily dose (mg/day).....Duration.....(years).....(months)

2. For what reason were you taking this statin?

_____

3. Which medical conditions did you have when taking statin? Please list them below:

_____

_____

_____

_____

_____

4. Were you taking other medications? ☐ Yes; ☐ No

If YES then please list them below:

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

Drug name ..... Daily dose (mg/day).....Duration .....(years).....(months)

---

5. Was your pain triggered or aggravated by? (Tick those that apply)

☐ Physical exercise ☐ Rest ☐ Cold ☐ Lying in a certain way ☐ Fasting

6. Would you describe your pain as? (Tick those that apply)

☐ Cramps
☐ Stiffness
☐ Heaviness
☐ Muscular weakness or decrease of muscle power during physical activity (like trouble rising from a chair or walking up or down stairs)
☐ Tendonitis (ache, pain or local stiffness surrounding a joint in arm or leg)
☐ Other (please specify) _____

---

7. What was the time to onset of muscle symptoms after starting statin therapy? (Please tick only one box:

☐ 1–2 days ☐ <1 month ☐ 1–3months ☐ 3–12 months ☐ >12 months

8. Would you describe the pain/weakness you suffered from, as?

☐ Permanent (all the time), or ☐ Intermittent (for hours or minutes)

9. Did you have muscle pain/weakness before you started on statin therapy?

☐ Yes ☐ No ☐ Not sure

10. Did you have any form of surgery whilst taking statins?

☐ Yes ☐ No ; If YES, what kind? _____

---

11. Is there any history of muscle disorders in your family or has any other member of your family has complained of muscle pain/weakness linked to taking statins?

☐ Yes; ☐ No

If YES then please give as much detail as you can below:

_____

_____

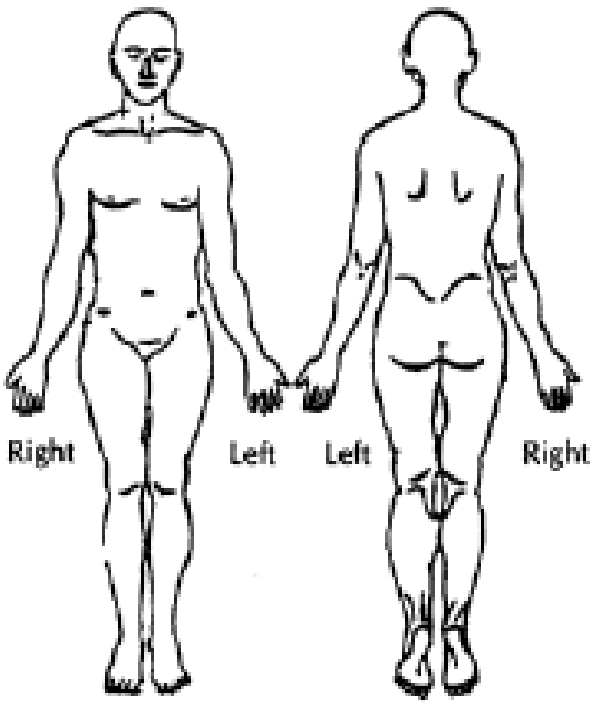
_____

_____

12. Were you taking painkillers to ease the pain caused by statin treatment?

☐ Yes ☐ No

13. On the diagram below shade the areas where you had pain or other muscle symptoms whilst on statin therapy. Put an X on the area which was affected most.



14. Please rate your pain by circling the number that best describes the pain you had on average whilst on statin therapy

0	1	2	3	4	5	6	7	8	9	10
No pain										Pain as bad as you can imagine

15. Please circle the number that best describes the pain you have right now

0	1	2	3	4	5	6	7	8	9	10
No pain										Pain as bad as you can imagine

16. Check the appropriate answer for each item.

I believe my pain (or muscle symptom) was due to:

a. The effect of treatment (for example, medication, radiation, surgery, prosthetic device)

☐ Yes ☐ No

b. My primary disease (meaning the disease currently being treated and evaluated)

☐ Yes ☐ No

c. A medical condition unrelated to my primary disease (for example arthritis)

☐ Yes ☐ No Please describe if answer is YES:_____

_____

17. Circle the one number that describes how during a typical week the pain (or muscle symptom) caused by statin therapy interfered with your:

A. General activity

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

B. Mood

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

C. Walking ability

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

D. Normal work (includes work both outside the house and housework)

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

E. Relations with other people

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

F. Sleep

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

G. Enjoyment of life

0	1	2	3	4	5	6	7	8	9	10
Does not interfere										Completely interferes

18. Do you exercise in addition to your routine activity on a regular weekly basis (including walking, jogging, gardening)?

☐ Yes ☐ No

19. With statin treatment withdrawal, did your pain disappear?

☐ (a)Completely ☐ (b)Partially ☐ (c) Not at all

If you ticked 'Partially' or 'Completely' approximately how long did it take for the pain to go away?

_____(days) or _____(months)

20. After stopping statin were you prescribed another lipid lowering drug?

☐ Yes ☐ No

If yes then please provide detail: Drug name ..... Daily dose (mg/day).....

21. Did muscle symptoms reappear with the new drug?

☐ Yes ☐ No

APPENDIX H Data presentations according to the total number of completed patient recruitment of 607 patients (125 cases and 482 controls).

1. Demographic and clinical characteristics of patients who were statin-tolerant (control) and those who withdrew statin therapy due to perceived muscle symptoms (SRM cases).

Variables	**Control (n=314)**	**Cases (n=116)**	**P-value**
Sex			**0.004***
Female, N (%)	213 (44.2%)	73 (58.4%	
Male, N (%)	269 (55.8%)	52 (41.6%)	
Age, mean years (± SD)	60.9 (± 13.2)	60.3 (± 9.1)	0.610
BMI, median (LQ, UQ)	28.5 (25.8, 32.0)	28.5 (26.4, 31.7)	0.933†
Duration on statin (months) median (LQ, UQ)	24 (12, 65)	8 (3, 18)	<0.001†
Statin, N (%)			
Simvastatin,	**297 (61.6)**	**103 (82.4)**	
10 mg/day	-	7 (6.8)	
20 mg/day	4 (1.3)	15 (14.6)	
40 mg/day	286 (96.3)	77 (74.8)	
80 mg/day	7 (2.4)	4 (3.9)	
Atorvastatin,	**184# (38.2)**	**17 (13.6)**	
10 mg/day	1 (0.5)	1(5.9)	
20 mg/day	2 (1.1)	-	
30 mg/day	1 (0.5)	-	
40 mg/day	12 (6.6)	1 (5.9)	
80 mg/day	167 (91.3)	15 (88.2)	
Rosuvastatin,	**1 (0.2)**	**5 (4)**	

*Pearson Chi-Square test (2-tailed) was performed for the analysis
†t-test after log transformation to achieve approximate normality
#one missing value from one individual in this group

2. Univariate analysis of the association between individual SNPs and statin-related muscle toxicity by univariate analysis.

Gene and SNP	Genotype	Frequencies, n		P-value for HWE[a]	P-value[b]	OR (95% CI)
		Control[c]	Cases			
SLCO1B1 rs4145096	WT Het Mut	354 112 11	78 45 2	0.547	**0.011**	1.73 (1.14- 2.64)
ABCC2 rs717620	WT Het Mut	301 158 18	80 39 6	0.626	0.917	0.962 (0.64 – 1.45)
ABCG2 rs2231142	WT Het Mut	375 94 7	104 19 2	0.690	0.318	0.8365 (0.49 -1.44)
CYP3A4 rs35599367 (*22)	WT Het Mut	423 51 2	111 14 0	0.729	1.000	1.01 (0.54 -1 .88)
COQ2 rs4693075	WT Het Mut	190 220 66	51 57 17	0.856	0.918	0.96 (0.64 – 1.44)
GATM rs9806699	WT Het Mut	252 176 47	64 48 13	0.052	0.763	1.07 (0.73 – 1.60)
GPx1 rs1050450	WT Het Mut	245 160 54	63 52 10	<0.001	ND	ND
GPx4 rs713041	WT Het Mut	137 251 85	39 59 27	0.106	0.739	1.09 (0.70-1.70)
SLC16A1 rs1049434	WT Het Mut	130 255 88	36 54 26	0.059	0.435	0.84 (0.54-1.28)
rs3849174	WT Het Mut	309 151 16	70 51 4	0.639	0.077	1.45 (0.97-2.17)

rs4301628	WT	206	63	0.437	0.159	0.75 (0.51-1.11)
	Het	220	52			
	Mut	50	10			
SLC16A3						
rs7503429	WT	130	36	0.797	0.823	0.95 (0.61-1.46)
	Het	237	82			
	Mut	103	7			
rs12453290	WT	217	64	0.128	0.269	0.76 (0.51-1.23)
	Het	220	47			
	Mut	40	14			

[a] P-value for HWE calculated in control group and compared with CEU population

[b] P-value derived from using Fisher's Exact test analysed between wild type individuals and combined heterozygous+homozygous mutant individuals in 2 x 2 tables.

[c] 3 missing values in control group

ND, not determined

3. Association between patient sex, rs4149056 SNP and SRM (adjusted for patient age, sex, BMI and other 11 genotypes) according to binary logistic regression analysis.

Dependent variable	Controls	Cases	Explanatory variables	P-value[#]	OR	95% CI for Odds Ratio¶	
						Lower	Upper
Statin-related muscle toxicity	482*	125	Patient sex	**0.006**	1.72	1.15	2.59
			SLCO1B1 (rs4149056)	**0.014**	1.66	1.08	2.54
			MRP2 (rs717620)	0.800	0.96	0.64	1.49
			BCRP1 (rs2231142)	0.228	0.74	0.44	1.25
			CYP3A4*22 (rs35599367)	0.884	0.98	0.52	1.86
			COQ2 (rs4693075)	0.837	0.98	0.65	1.48
			GATM (rs9806699)	0.747	1.03	0.68	1.54
			GPx4 (rs703041)	0.733	1.09	0.69	1.70
			MCT1 (rs1049434)	0.456	0.83	0.49	1.44
			MCT1 (rs3849174)	0.121	1.29	0.78	2.04
			MCT1(rs4301628)	0.406	0.80	0.49	1.33
			MCT4 (rs7503429)	0.990	0.95	0.61	1.51
			MCT4 (rs12453290)	0.310	0.81	0.54	1.22

* Genotypes from 5 individuals were not included in the analysis.

[#] P-value for comparison of genotypes frequencies possession of one or two variant alleles between cases vs controls (reference) using multiple linear regression; Hosmer and Lemeshow goodness-of-fit test was used as an indicator of the validity at the last step of iterations

¶ Odds ratio describes the odds to have at least one variant allele in the cases relative to the controls.

4. The resultant OR relative to male wild type (TT genotypes) among rs4149056 genotypes

Category	**OR**	**95 % CI**	**P-value**
Male TC/CC genotypes	2.43	1.3140, 4.4843	0.0046
Female TT genotypes	2.25	1.3550, 3.7212	0.0017
Female TC/CC genotypes	2.93	1.5847, 5.4279	0.0006

5. Stratified analyses for the effect of patient sex and BMI according to the rs4149056 genotypes

Parameter	**Study cohort**	**Frequency of rs4149056 genotypes**		**P-value**
		Wild type, n	**C allele carriers, n**	
BMI	Controls		123 (mean logged BMI= 3.382)	**0.02**
	Cases		47 (mean logged BMI= 3.320)	
BMI	Controls	354 (mean logged BMI=3.358)		0.13
	Cases	78 (mean logged BMI= 3.391)		
Sex	Controls		66 (Male) 57 (Female)	0.581
	Cases		23 (Male) 24 (Female)	
Sex	Controls	202 (Male) 152 (Female)		**0.001**
	Cases	29 (Male) 49 (Female)		

Chapter 7. References

Abd, T.T. and Jacobson, T.A. (2011) 'Statin-induced myopathy: a review and update', *Expert Opin Drug Saf*, 10(3), pp. 373-87.

Adijanto, J. and Philp, N. (2012) 'The SLC16A family of monocarboxylate transporters (MCTs)--physiology and function in cellular metabolism, pH homeostasis, and fluid transport', *Curr Top Membr*, 70, pp. 275-311.

Agbabiaka, T.B., Savovic, J. and Ernst, E. (2008) 'Methods for causality assessment of adverse drug reactions: a systematic review', *Drug Saf*, 31(1), pp. 21-37.

Alagona, P., Jr. (2010) 'Pitavastatin: evidence for its place in treatment of hypercholesterolemia', *Core Evid*, 5, pp. 91-105.

Alfirevic, A., Neely, D., Armitage, J., Chinoy, H., Cooper, R. G., Laaksonen, R., Carr, D. F., Bloch, K. M., Fahy, J., Hanson, A., Yue, Q. Y., Wadelius, M., Maitland-van Der Zee, A. H., Voora, D., Psaty, B. M., Palmer, C. N. and Pirmohamed, M. (2014) 'Phenotype standardization for statin-induced myotoxicity', *Clin Pharmacol Ther*, 96(4), pp. 470-6.

Altshuler, D. M., Gibbs, R. A., Peltonen, L., Altshuler, D. M. and al, e. (2010) 'Integrating common and rare genetic variation in diverse human populations', *Nature*, 467(7311), pp. 52-8.

Amarenco, P., Bogousslavsky, J., Callahan, A., 3rd, Goldstein, L. B., Hennerici, M., Rudolph, A. E., Sillesen, H., Simunovic, L., Szarek, M., Welch, K. M. and Zivin, J. A. (2006) 'High-dose atorvastatin after stroke or transient ischemic attack', *N Engl J Med*, 355(6), pp. 549-59.

Ambudkar, S.V., Kimchi-Sarfaty, C., Sauna, Z.E. and Gottesman, M.M. (2003) 'P-glycoprotein: from genomics to mechanism', *Oncogene*, 22(47), pp. 7468-85.

Ansari, A.-H. (2007) *Targeting statins to the endothelial cells*. University of Newcastle upon Tyne.

Armitage, J. (2007) 'The safety of statins in clinical practice', *Lancet*, 370(9601), pp. 1781-90.

Athyros, V. G., Tziomalos, K., Karagiannis, A. and Mikhailidis, D. P. (2010) 'Atorvastatin: safety and tolerability', *Expert Opin Drug Saf*, 9(4), pp. 667-74.

Austin, M. A., Hutter, C. M., Zimmern, R. L. and Humphries, S. E. (2004) 'Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review', *Am J Epidemiol*, 160(5), pp. 407-20.

Ayrton, A. and Morgan, P. (2001) 'Role of transport proteins in drug absorption, distribution and excretion', *Xenobiotica*, 31(8-9), pp. 469-97.

Azie, N. E., Brater, D. C., Becker, P. A., Jones, D. R. and Hall, S. D. (1998) 'The interaction of diltiazem with lovastatin and pravastatin', *Clin Pharmacol Ther*, 64(4), pp. 369-77.

Backes, J. M. and Howard, P. A. (2003) 'Association of HMG-CoA reductase inhibitors with neuropathy', *Ann Pharmacother*, 37(2), pp. 274-8.

Bailey, K. M., Romaine, S. P., Jackson, B. M., Farrin, A. J., Efthymiou, M., Barth, J. H., Copeland, J., McCormack, T., Whitehead, A., Flather, M. D., Samani, N. J., Nixon, J., Hall, A. S. and Balmforth, A. J. (2010) 'Hepatic metabolism and transporter gene variants enhance response to rosuvastatin in patients with acute myocardial infarction: the GEOSTAT-1 Study', *Circ Cardiovasc Genet*, 3(3), pp. 276-85.

Ballantyne, C.M., Herd, J.A., Stein, E.A., Ferlic, L.L., Dunn, J.K., Gotto, A.M., Jr. and Marian, A.J. (2000) 'Apolipoprotein E genotypes and response of plasma lipids and progression-regression of coronary atherosclerosis to lipid-lowering drug therapy', *J Am Coll Cardiol*, 36(5), pp. 1572-8.

Ballantyne, C. M., Corsini, A., Davidson, M. H., Holdaas, H., Jacobson, T. A., Leitersdorf, E., Marz, W., Reckless, J. P. and Stein, E. A. (2003) 'Risk for myopathy with statin therapy in high-risk patients', *Arch Intern Med*, 163(5), pp. 553-64.

Ballard, K. D. and Thompson, P. D. (2013) 'Does reduced creatine synthesis protect against statin myopathy?', *Cell Metab*, 18(6), pp. 773-4.

Ballatori, N., Hammond, C. L., Cunningham, J. B., Krance, S. M. and Marchan, R. (2005) 'Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins', *Toxicol Appl Pharmacol*, 204(3), pp. 238-55.

Bays, H. (2006) 'Statin safety: an overview and assessment of the data--2005', *Am J Cardiol*, 97(8a), pp. 6c-26c.

Becker, M. L., Elens, L. L., Visser, L. E., Hofman, A., Uitterlinden, A. G., van Schaik, R. H. and Stricker, B. H. (2013) 'Genetic variation in the ABCC2 gene is associated with dose decreases or switches to other cholesterol-lowering drugs during simvastatin and atorvastatin therapy', *Pharmacogenomics J*, 13(3), pp. 251-6.

Bellosta, S., Paoletti, R. and Corsini, A. (2004) 'Safety of statins: focus on clinical pharmacokinetics and drug interactions', *Circulation*, 109(23 Suppl 1), pp. Iii50-7.

Blau, H. M. and Webster, C. (1981) 'Isolation and characterization of human muscle cells', *Proc Natl Acad Sci U S A*, 78(9), pp. 5623-7.

Blein, S., Berndt, S., Joshi, A. D., Campa, D., Ziegler, R. G., Riboli, E. and Cox, D. G. (2014) 'Factors associated with oxidative stress and cancer risk in the Breast and Prostate Cancer Cohort Consortium', *Free Radic Res*, 48(3), pp. 380-6.

Bonen, A. (2001) 'The expression of lactate transporters (MCT1 and MCT4) in heart and muscle', *European Journal of Applied Physiology*, 86(1), pp. 6-11.

Bonen, A., Baker, S. K. and Hatta, H. (1997) 'Lactate transport and lactate transporters in skeletal muscle', *Can J Appl Physiol*, 22(6), pp. 531-52.

Bonen, A., Tonouchi, M., Miskovic, D., Heddle, C., Heikkila, J. J. and Halestrap, A. P. (2000) 'Isoform-specific regulation of the lactate transporters MCT1 and MCT4 by contractile activity', *Am J Physiol Endocrinol Metab*, 279(5), pp. E1131-8.

Bonetti, P. O., Lerman, L. O., Napoli, C. and Lerman, A. (2003) 'Statin effects beyond lipid lowering--are they clinically relevant?', *Eur Heart J*, 24(3), pp. 225-48.

Bonetti, P. O., Wilson, S. H., Rodriguez-Porcel, M., Holmes, D. R., Jr., Lerman, L. O. and Lerman, A. (2002) 'Simvastatin preserves myocardial perfusion and coronary microvascular permeability in experimental hypercholesterolemia independent of lipid lowering', *J Am Coll Cardiol*, 40(3), pp. 546-54.

Bonifacio, A., Mullen, P. J., Mityko, I. S., Navegantes, L. C., Bouitbir, J. and Krahenbuhl, S. (2014) 'Simvastatin induces mitochondrial dysfunction and increased atrogin-1 expression in H9c2 cardiomyocytes and mice in vivo', *Arch Toxicol*.

Bottorff, M. B. (2006) 'Statin safety and drug interactions: clinical implications', *Am J Cardiol*, 97(8a), pp. 27c-31c.

Bouitbir, J., Charles, A. L., Rasseneur, L., Dufour, S., Piquard, F., Geny, B. and Zoll, J. (2011) 'Atorvastatin treatment reduces exercise capacities in rats: involvement of mitochondrial impairments and oxidative stress', *J Appl Physiol (1985)*, 111(5), pp. 1477-83.

Broer, S., Schneider, H. P., Broer, A., Rahman, B., Hamprecht, B. and Deitmer, J. W. (1998) 'Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH', *Biochem J*, 333 (Pt 1), pp. 167-74.

Brooks, G. A. (2009) 'Cell-cell and intracellular lactate shuttles', *J Physiol*, 587(Pt 23), pp. 5591-600.

Brooks, G. A., Brown, M. A., Butz, C. E., Sicurello, J. P. and Dubouchaud, H. (1999) 'Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1', *J Appl Physiol (1985)*, 87(5), pp. 1713-8.

Brown, A. E., Yeaman, S. J. and Walker, M. (2007) 'Targeted suppression of calpain-10 expression impairs insulin-stimulated glucose uptake in cultured primary human skeletal muscle cells', *Mol Genet Metab*, 91(4), pp. 318-24.

Brown, M. S. and Goldstein, J. L. (1986) 'A receptor-mediated pathway for cholesterol homeostasis', *Science*, 232(4746), pp. 34-47.

Bruckert, E., Hayem, G., Dejager, S., Yau, C. and Begaud, B. (2005) 'Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients--the PRIMO study', *Cardiovasc Drugs Ther*, 19(6), pp. 403-14.

Brugts, J. J., Yetgin, T., Hoeks, S. E., Gotto, A. M., Shepherd, J., Westendorp, R. G., de Craen, A. J., Knopp, R. H., Nakamura, H., Ridker, P., van Domburg, R. and Deckers, J. W. (2009) 'The benefits of statins in people without established cardiovascular disease but with cardiovascular risk factors: meta-analysis of randomised controlled trials', *Bmj*, 338, p. b2376.

Bruhn, O. and Cascorbi, I. (2014) 'Polymorphisms of the drug transporters ABCB1, ABCG2, ABCC2 and ABCC3 and their impact on drug bioavailability and clinical relevance', *Expert Opin Drug Metab Toxicol*, 10(10), pp. 1337-54.

Brunham, L. R., Lansberg, P. J., Zhang, L., Miao, F., Carter, C., Hovingh, G. K., Visscher, H., Jukema, J. W., Stalenhoef, A. F., Ross, C. J. D., Carleton, B. C., Kastelein, J. J. P. and Hayden, M. R. (2012) 'Differential effect of the rs4149056 variant in SLCO1B1 on myopathy associated with simvastatin and atorvastatin', *The Pharmacogenomics Journal* 12, pp. 233-237.

Buchler, M., Konig, J., Brom, M., Kartenbeck, J., Spring, H., Horie, T. and Keppler, D. (1996) 'cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats', *J Biol Chem*, 271(25), pp. 15091-8.

Burnell, J. M. and Kirby, W. M. (1951) 'Effectiveness of a new compound, benemid, in elevating serum penicillin concentrations', *J Clin Invest*, 30(7), pp. 697-700.

Butz, C. E., McClelland, G. B. and Brooks, G. A. (2004) 'MCT1 confirmed in rat striated muscle mitochondria. ', *J Appl Physiol*, 97, pp. 1059–1066.

Byng, M. C., Whittaker, J. C., Cuthbert, A. P., Mathew, C. G. and Lewis, C. M. (2003) 'SNP subset selection for genetic association studies', *Ann Hum Genet*, 67(Pt 6), pp. 543-56.

Canestaro, W. J., Austin, M. A. and Thummel, K. E. (2014) 'Genetic factors affecting statin concentrations and subsequent myopathy: a HuGENet systematic review', *Genet Med*, 16(11), pp. 810-9.

Cannon, C. P., Braunwald, E., McCabe, C. H. and al., e. (2004) 'Intensive versus moderate lipid lowering with statins after acute coronary syndromes. ', *N Eng J Med*, 350, pp. 1495-1504.

Carlson, C. S., Eberle, M. A., Rieder, M. J., Yi, Q., Kruglyak, L. and Nickerson, D. A. (2004) 'Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium', *Am J Hum Genet*, 74(1), pp. 106-20.

Carr, D. F., O'Meara, H., Jorgensen, A. L., Campbell, J., Hobbs, M., McCann, G., van Staa, T. and Pirmohamed, M. (2013) 'SLCO1B1 genetic variant associated with statin-induced myopathy: a proof-of-concept study using the clinical practice research datalink', *Clin Pharmacol Ther*, 94(6), pp. 695-701.

Ceckova, M., Libra, A., Pavek, P., Nachtigal, P., Brabec, M., Fuchs, R. and Staud, F. (2006) 'Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo', *Clin Exp Pharmacol Physiol*, 33(1-2), pp. 58-65.

Chariot, P., Abadia, R., Agnus, D., Danan, C., Charpentier, C. and Gherardi, R. K. (1993) 'Simvastatin-induced rhabdomyolysis followed by a MELAS syndrome', *Am J Med*, 94(1), pp. 109-10.

Chatzizisis, Y. S., Koskinas, K. C., Misirli, G., Vaklavas, C., Hatzitolios, A. and Giannoglou, G. D. (2010) 'Risk Factors and Drug Interactions Predisposing to Statin-Induced Myopathy Implications for Risk Assessment, Prevention and Treatment', *Drug safety*, 33(3), pp. 171-187.

Cheng, Y. and Prusoff, W. H. (1973) 'Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction', *Biochem Pharmacol*, 22(23), pp. 3099-108.

Christians, U., Jacobsen, W. and Floren, L. C. (1998) 'Metabolism and drug interactions of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in transplant patients: are the statins mechanistically similar?', *Pharmacol Ther*, 80(1), pp. 1-34.

Cleeman, J., Grundy, S., Becker, D., Clark, L., Cooper, R., Denke, M., Howard, W., Hunninghake, D., Illingworth, D., Luepker, R., Mcbride, P., Mckenney, J., Pasternak, R., Stone, N., Van Horn, L., Brewer, H., Ernst, N., Gordon, D., Levy, D., Rifkind, B., Rossouw, J., Savage, P., Haffner, S., Orloff, D., Proschan, M., Schwartz, J., Sempos, C., Shero, S. and Murray, E. (2001) 'Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III)', *Journal of the American Medical Association*, 285(19), pp. 2486-97.

Coleman, C. I., Reinhart, K., Kluger, J. and White, C. M. (2008) 'The effect of statins on the development of new-onset type 2 diabetes: a meta-analysis of randomized controlled trials', *Curr Med Res Opin*, 24(5), pp. 1359-62.

Copaja, M., Venegas, D., Aranguiz, P., Canales, J., Vivar, R., Catalan, M., Olmedo, I., Rodriguez, A. E., Chiong, M., Leyton, L., Lavandero, S. and Diaz-Araya, G. (2011) 'Simvastatin induces apoptosis by a Rho-dependent mechanism in cultured cardiac fibroblasts and myofibroblasts', *Toxicol Appl Pharmacol*, 255(1), pp. 57-64.

Corsini, A. (2003) 'The safety of HMG-CoA reductase inhibitors in special populations at high cardiovascular risk', *Cardiovasc Drugs Ther*, 17(3), pp. 265-85.

Corsini, A., Bellosta, S., Baetta, R., Fumagalli, R., Paoletti, R. and Bernini, F. (1999) 'New insights into the pharmacodynamic and pharmacokinetic properties of statins', *Pharmacol Ther*, 84(3), pp. 413-28.

Cousin, E., Genin, E., Mace, S., Ricard, S., Chansac, C., del Zompo, M. and Deleuze, J. F. (2003) 'Association studies in candidate genes: strategies to select SNPs to be tested', *Hum Hered*, 56(4), pp. 151-9.

Crissman, H. A. and Steinkamp, J. A. (1987) 'A new method for rapid and sensitive detection of bromodeoxyuridine in DNA-replicating cells', *Exp Cell Res*, 173(1), pp. 256-61.

Cziraky, M.J., Willey, V.J., McKenney, J.M., Kamat, S.A., Fisher, M.D., Guyton, J.R., Jacobson, T.A. and Davidson, M.H. (2006) 'Statin safety: an assessment using an administrative claims database', *Am J Cardiol*, 97(8a), pp. 61c-68c.

Daly, A. K. and Day, C. P. (2001) 'Candidate gene case-control association studies: advantages and potential pitfalls', *Br J Clin Pharmacol*, 52(5), pp. 489-99.

Daly, A. K., Monkman, S. C., Smart, J., Steward, A. and Cholerton, S. (1998) 'Analysis of cytochrome P450 polymorphisms', *Methods Mol Biol*, 107, pp. 405-22.

Davidson, M. H., Stein, E. A., Dujovne, C. A., Hunninghake, D. B., Weiss, S. R., Knopp, R. H., Illingworth, D. R., Mitchel, Y. B., Melino, M. R., Zupkis, R. V. and al., e. (1997) 'The efficacy and six-week tolerability of simvastatin 80 and 160 mg/day. ', *79*, pp. 38-42.

De Backer, G., Ambrosioni, E., Borch-Johnsen, K., Brotons, C., Cifkova, R., Dallongeville, J., Ebrahim, S., Faergeman, O., Graham, I., Mancia, G., Cats, V. M., Orth-Gomer, K., Perk, J., Pyorala, K., Rodicio, J. L., Sans, S., Sansoy, V., Sechtem, U., Silber, S., Thomsen, T. and Wood, D. (2003) 'European guidelines

on cardiovascular disease and prevention in clinical practice', *Atherosclerosis*, 171(1), pp. 145-55.

de Graan, A. J., Lancaster, C. S., Obaidat, A., Hagenbuch, B., Elens, L., Friberg, L. E., de Bruijn, P., Hu, S., Gibson, A. A., Bruun, G. H., Corydon, T. J., Mikkelsen, T. S., Walker, A. L., Du, G., Loos, W. J., van Schaik, R. H., Baker, S. D., Mathijssen, R. H. and Sparreboom, A. (2012) 'Influence of polymorphic OATP1B-type carriers on the disposition of docetaxel', *Clin Cancer Res*, 18(16), pp. 4433-40.

de Lemos, J. A., Blazing, M. A., Wiviott, S. D., Lewis, E. F., Fox, K. A., White, H. D., Rouleau, J. L., Pedersen, T. R., Gardner, L. H., Mukherjee, R., Ramsey, K. E., Palmisano, J., Bilheimer, D. W., Pfeffer, M. A., Califf, R. M. and Braunwald, E. (2004) 'Early intensive vs a delayed conservative simvastatin strategy in patients with acute coronary syndromes: phase Z of the A to Z trial', *Jama*, 292(11), pp. 1307-16.

Deedwania, P., Stone, P. H., Bairey Merz, C. N., Cosin-Aguilar, J., Koylan, N., Luo, D., Ouyang, P., Piotrowicz, R., Schenck-Gustafsson, K., Sellier, P., Stein, J. H., Thompson, P. L. and Tzivoni, D. (2007) 'Effects of intensive versus moderate lipid-lowering therapy on myocardial ischemia in older patients with coronary heart disease: results of the Study Assessing Goals in the Elderly (SAGE)', *Circulation*, 115(6), pp. 700-7.

Di Rosa, E. and Di Rosa, A. E. (2014) 'Loperamide overdose-induced catatonia: potential role of brain opioid system and P-glycoprotein', *Acta Neuropsychiatr*, 26(1), pp. 58-60.

Dickhout, J. G., Hossain, G. S., Pozza, L. M., Zhou, J., Lhotak, S. and Austin, R. C. (2005) 'Peroxynitrite causes endoplasmic reticulum stress and apoptosis in human vascular endothelium: implications in atherogenesis', *Arterioscler Thromb Vasc Biol*, 25(12), pp. 2623-9.

Dirks, A. J. and Jones, K. M. (2006) 'Statin-induced apoptosis and skeletal myopathy', *Am J Physiol Cell Physiol*, 291(6), pp. C1208-12.

Donnelly, L. A., Doney, A. S., Tavendale, R., Lang, C. C., Pearson, E. R., Colhoun, H. M., McCarthy, M. I., Hattersley, A. T., Morris, A. D. and Palmer, C. N. (2011) 'Common nonsynonymous substitutions in SLCO1B1 predispose to statin intolerance in routinely treated individuals with type 2 diabetes: a go-DARTS study', *Clin Pharmacol Ther*, 89(2), pp. 210-6.

Dorajoo, R., Pereira, B. P., Yu, Z., Gopalakrishnakone, P., Leong, C. C., Wee, A. and Lee, E. (2008) 'Role of multi-drug resistance-associated protein-1 transporter in statin-induced myopathy', *Life Sci*, 82(15-16), pp. 823-30.

Dorak, M.T. (2006) *Real-time PCR*. Cornwall, United Kingdom: Taylor & Francis Group.

Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W. and Gotto, A. M., Jr. (1998) 'Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study', *Jama*, 279(20), pp. 1615-22.

El Abida, K., Duvallet, A., Thieulart, L., Rieu, M. and Beaudry, M. (1992) 'Lactate transport during differentiation of skeletal muscle cells: evidence for a specific carrier in L6 myotubes ', *Acta Physiol Scand*, 144, pp. 469-471.

Elens, L., Becker, M. L., Haufroid, V., Hofman, A., Visser, L. E., Uitterlinden, A. G., Stricker, B. and van Schaik, R. H. (2011) 'Novel CYP3A4 intron 6 single nucleotide polymorphism is associated with simvastatin-mediated cholesterol reduction in the Rotterdam Study', *Pharmacogenet Genomics*, 21(12), pp. 861-6.

Elens, L., van Gelder, T., Hesselink, D. A., Haufroid, V. and van Schaik, R. H. (2013) 'CYP3A4*22: promising newly identified CYP3A4 variant allele for personalizing pharmacotherapy.', *Pharmacogenomics* 14(1), pp. 47-62.

Elghannam, H., Tavackoli, S., Ferlic, L., Gotto, A.M., Jr., Ballantyne, C.M. and Marian,

A.J. (2000) 'A prospective study of genetic markers of susceptibility to infection and inflammation, and the severity, progression, and regression of coronary atherosclerosis and its response to therapy', *J Mol Med (Berl)*, 78(10), pp. 562-8.

Ellis, L. C., Hawksworth, G. M. and Weaver, R. J. (2013) 'ATP-dependent transport of statins by human and rat MRP2/Mrp2', *Toxicol Appl Pharmacol*, 269(2), pp. 187-94.

El-Salem, K., Ababneh, B., Rudnicki, S., Malkawi, A., Alrefai, A., Khader, Y., Saadeh, R. and Saydam, M. (2011) 'Prevalence and risk factors of muscle complications secondary to statins.', *Muscle Nerve*, 44(6), pp. 877-881.

Evans, M. A. and Rees, A. (2002) 'Effects of HMG-CoA reductase inhibitors on skeletal muscle: are all statins the same?', *Drug Saf*, 25(9), pp. 649-663.

Falconer, D.S. and Mackay, T.F.C. (1996) *Introduction to Quantitative Genetics*. New York: Longman.

Fang, S. Y., Roan, J. N., Luo, C. Y., Tsai, Y. C. and Lam, C. F. (2013) 'Pleiotropic vascular protective effects of statins in perioperative medicine', *Acta Anaesthesiol Taiwan*, 51(3), pp. 120-6.

Farmer, J. A. (2000) 'Pleiotropic effects of statins', *Curr Atheroscler Rep*, 2(3), pp. 208-17.

Feng, Q., Wilke, R. A. and Baye, T. M. (2012) 'Individualized risk for statin-induced myopathy: current knowledge, emerging challenges and potential solutions', *Pharmacogenomics*, 13(5), pp. 579-94.

Fernandes e Fernandes, J., Pedro, M., Fernandes eFernandes, R. and Silvestre, L. (2014) 'Imaging Occlusive Atherosclerosis', in Saba, L. and et al (eds.) *Multi-Modality Atherosclerosis Imaging and Diagnosis*. Lisbon, Portugal: Springer Science and Business Media.

Ferrari, M., Guasti, L., Maresca, A., Mirabile, M., Contini, S., Grandi, A. M., Marino, F. and Cosentino, M. (2014) 'Association between statin-induced creatine kinase elevation and genetic polymorphisms in SLCO1B1, ABCB1 and ABCG2', *Eur J Clin Pharmacol*, 70(5), pp. 539-47.

Fichtenbaum, C. J., Gerber, J. G., Rosenkranz, S. L., Segal, Y., Aberg, J. A., Blaschke, T., Alston, B., Fang, F., Kosel, B. and Aweeka, F. (2002) 'Pharmacokinetic interactions between protease inhibitors and statins in HIV seronegative volunteers: ACTG Study A5047', *Aids*, 16(4), pp. 569-77.

Fiegenbaum, M., da Silveira, F.R., Van der Sand, C.R., Van der Sand, L.C., Ferreira, M.E., Pires, R.C. and Hutz, M.H. (2005) 'The role of common variants of ABCB1, CYP3A4, and CYP3A5 genes in lipid-lowering efficacy and safety of simvastatin treatment', *Clin Pharmacol Ther*, 78(5), pp. 551-8.

Folkers, K., Langsjoen, P., Willis, R., Richardson, P., Xia, L. J., Ye, C. Q. and Tamagawa, H. (1990) 'Lovastatin decreases coenzyme Q levels in humans', *Proc Natl Acad Sci U S A*, 87(22), pp. 8931-4.

Forrester, J. S. and Libby, P. (2007) 'The inflammation hypothesis and its potential relevance to statin therapy', *Am J Cardiol*, 99(5), pp. 732-8.

Forster, F., Volz, A. and Fricker, G. (2008) 'Compound profiling for ABCC2 (MRP2) using a fluorescent microplate assay system', *Eur J Pharm Biopharm*, 69(1), pp. 396-403.

Fromm, M.F. (2002) 'The influence of MDR1 polymorphisms on P-glycoprotein expression and function in humans', *Adv Drug Deliv Rev*, 54(10), pp. 1295-310.

Fujino, H., Saito, T., Ogawa, S. and Kojima, J. (2005) 'Transporter-mediated influx and efflux mechanisms of pitavastatin, a new inhibitor of HMG-CoA reductase', *J Pharm Pharmacol*, 57(10), pp. 1305-11.

Gadbut, A. P., Caruso, A. P. and Galper, J. B. (1995) 'Differential sensitivity of C2-C12 striated muscle cells to lovastatin and pravastatin. ', *J Mol Cell Cardiol*, 27(10), pp. 2397–2402.

Galtier, F., Mura, T., Raynaud de Mauverger, E., Chevassus, H., Farret, A., Gagnol, J. P., Costa, F., Dupuy, A., Petit, P., Cristol, J. P., Mercier, J. and Lacampagne, A. (2012) 'Effect of a high dose of simvastatin on muscle mitochondrial metabolism and calcium signaling in healthy volunteers', *Toxicol Appl Pharmacol*, 263(3), pp. 281-6.

Gautrey, H., Nicol, F., Sneddon, A. A., Hall, J. and Hesketh, J. (2011) 'A T/C polymorphism in the GPX4 3'UTR affects the selenoprotein expression pattern and cell viability in transfected Caco-2 cells', *Biochim Biophys Acta*, 1810(6), pp. 584-91.

Generaux, G. T., Bonomo, F. M., Johnson, M. and Doan, K. M. (2011) 'Impact of SLCO1B1 (OATP1B1) and ABCG2 (BCRP) genetic polymorphisms and inhibition on LDL-C lowering and myopathy of statins.', *Xenobiotica*, 41(8), pp. 639-651.

Giacomini, K. M., Balimane, P. V., Cho, S. K., Eadon, M., Edeki, T., Hillgren, K. M., Huang, S. M., Sugiyama, Y., Weitz, D., Wen, Y., Xia, C. Q., Yee, S. W., Zimdahl, H. and Niemi, M. (2013) 'International Transporter Consortium commentary on clinically important transporter polymorphisms', *Clin Pharmacol Ther*, 94(1), pp. 23-6.

Giannakopoulou, E., Ragia, G., Kolovou, V., Tavridou, A., Tselepis, A. D., Elisaf, M., Kolovou, G. and Manolopoulos, V. G. (2014) 'No impact of SLCO1B1 521T>C, 388A>G and 411G>A polymorphisms on response to statin therapy in the Greek population', *Mol Biol Rep*, 41(7), pp. 4631-8.

Ginsberg, H. N., Le, N. A., Short, M. P., Ramakrishnan, R. and Desnick, R. J. (1987) 'Suppression of apolipoprotein B production during treatment of cholesteryl ester storage disease with lovastatin. Implications for regulation of apolipoprotein B synthesis', *J Clin Invest*, 80(6), pp. 1692-7.

Gladden, L. B. (2004) 'Lactate metabolism: a new paradigm for the third millennium', *The Journal of Physiology*, 558(1), pp. 5-30.

Goldstein, J.L. and Brown, M.S. (1990) 'Regulation of the mevalonate pathway', *Nature*, 343(6257), pp. 425-30.

Goldstein, J. K., Hobbs, H. H. and Brown, M. S. (2001) *Familial hypercholesterolemia*. New York: McGraw-Hill.

Golomb, B. A. and Evans, M. A. (2008) 'Statin adverse effects: a review of the literature and evidence for a mitochondrial mechanism', *Am J Cardiovasc Drugs*, 8(6), pp. 373-418.

Gottesman, M. M. and Ambudkar, S. V. (2001) 'Overview: ABC transporters and human disease', *J Bioenerg Biomembr*, 33(6), pp. 453-8.

Graham, D.J., Staffa, J.A., Shatin, D., Andrade, S.E., Schech, S.D., La Grenade, L., Gurwitz, J.H., Chan, K.A., Goodman, M.J. and Platt, R. (2004) 'Incidence of hospitalized rhabdomyolysis in patients treated with lipid-lowering drugs', *Jama*, 292(21), pp. 2585-90.

Grundy, S. M. (1998) 'Consensus statement: Role of therapy with "statins" in patients with hypertriglyceridemia', *Am J Cardiol*, 81(4a), pp. 1b-6b.

Halestrap, A. P. and Price, N. T. (1999) 'The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation', *Biochem J*, 343 Pt 2, pp. 281-99.

Halldorsson, B. V., Bafna, V., Lippert, R., Schwartz, R., De La Vega, F. M., Clark, A. G. and Istrail, S. (2004) 'Optimal haplotype block-free selection of tagging SNPs for genome-wide association studies', *Genome Res*, 14(8), pp. 1633-40.

Hamelin, B. A. and Turgeon, J. (1998) 'Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. ', *Trends Pharmacol Sci* 19(1), pp. 26-37.

Han, J. Y., Lim, H. S., Yoo, Y. K., Shin, E. S., Park, Y. H., Lee, S. Y., Lee, J. E., Lee, D. H., Kim, H. T. and Lee, J. S. (2007) 'Associations of ABCB1, ABCC2, and ABCG2 polymorphisms with irinotecan-pharmacokinetics and clinical outcome in patients with advanced non-small cell lung cancer', *Cancer*, 110(1), pp. 138-47.

Hashimoto, T. and Brooks, G. A. (2008) 'Mitochondrial lactate oxidation complex and an adaptive role for lactate production', *Med Sci Sports Exerc*, 40(3), pp. 486-94.

Hashimoto, T., Hussien, R. and Brooks, G. A. (2006) 'Colocalization of MCT1, CD147, and LDH in mitochondrial inner membrane of L6 muscle cells: evidence of a mitochondrial lactate oxidation complex', *Am J Physiol Endocrinol Metab*, 290(6), pp. E1237-E1244.

Hashimoto, T., Hussien, R., Oommen, S., Gohil, K. and Brooks, G. A. (2007) 'Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis', *Faseb j*, 21(10), pp. 2602-12.

Heart Protection Study Collaborative Group (2002) 'MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial', *Lancet*, 360(9326), pp. 23-33.

Hillgren, K. M., Keppler, D., Zur, A. A., Giacomini, K. M., Stieger, B., Cass, C. E. and Zhang, L. (2013) 'Emerging transporters of clinical importance: an update from the International Transporter Consortium', *Clin Pharmacol Ther*, 94(1), pp. 52-63.

Hippisley-Cox, J. and Coupland, C. (2010) 'Individualising the risks of statins in men and women in England and Wales: population-based cohort study', *Heart*, 96(12), pp. 939-47.

Hirano, M., Maeda, K., Matsushima, S., Nozaki, Y., Kusuhara, H. and Sugiyama, Y. (2005) 'Involvement of BCRP (ABCG2) in the biliary excretion of pitavastatin', *Mol Pharmacol*, 68(3), pp. 800-7.

Ho, R. H., Tirona, R. G., Leake, B. F., Glaeser, H., Lee, W., Lemke, C. J., Wang, Y. and Kim, R. B. (2006) 'Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics', *Gastroenterology*, 130(6), pp. 1793-806.

Hong, Z., Tian, C. and Zhang, X. (2013) 'GPX1 gene Pro200Leu polymorphism, erythrocyte GPX activity, and cancer risk', *Mol Biol Rep*, 40(2), pp. 1801-12.

Hooijberg, J. H., Peters, G. J., Kaspers, G. J., Wielinga, P. R., Veerman, A. J., Pieters, R. and Jansen, G. (2004) 'Online fluorescent method to assess BCRP/ABCG2 activity in suspension cells', *Nucleosides Nucleotides Nucleic Acids*, 23(8-9), pp. 1451-4.

Hou, Q., Li, S., Li, L., Li, Y., Sun, X. and Tian, H. (2015) 'Association Between SLCO1B1 Gene T521C Polymorphism and Statin-Related Myopathy Risk: A Meta-Analysis of Case-Control Studies', *Medicine (Baltimore)*, 94(37), p. e1268.

Hu, M., To, K. K., Mak, V. W. and Tomlinson, B. (2011) 'The ABCG2 transporter and its relations with the pharmacokinetics, drug interaction and lipid-lowering effects of statins', *Expert Opin Drug Metab Toxicol*, 7(1), pp. 49-62.

Huerta-Alardin, A., Varon, J. and Marik, P. (2005) 'Bench-to-bedside review: Rhabdomyolysis – an overview for clinicians', *Crit Care*, 9, pp. 158-169.

Imaoka, T., Mikkaichi, T., Abe, K., Hirouchi, M., Okudaira, N. and Izumi, T. (2013) 'Integrated approach of *in vivo* and *in vitro* evaluation of the involvement of hepatic uptake organic anion transporters in the drug disposition in rats using rifampicin as an inhibitor', *Drug Metab Dispos*, 41(7), pp. 1442-9.

Ishigami, M., Tokui, T., Komai, T., Tsukahara, K., Yamazaki, M. and Sugiyama, Y. (1995) 'Evaluation of the uptake of pravastatin by perfused rat liver and primary cultured rat hepatocytes', *Pharm Res*, 12(11), pp. 1741-5.

Itagaki, S., Chiba, M., Kobayashi, M., Hirano, T. and Iseki, K. (2008) 'Contribution of multidrug resistance-associated protein 2 to secretory intestinal transport of organic anions', *Biol Pharm Bull*, 31(1), pp. 146-8.

Jacobson, T. A. (2004) 'Comparative pharmacokinetic interaction profiles of pravastatin, simvastatin, and atorvastatin when coadministered with cytochrome P450 inhibitors', *Am J Cardiol*, 94(9), pp. 1140-6.

Jemnitz, K., Veres, Z., Tugyi, R. and Vereczkey, L. (2010) 'Biliary efflux transporters involved in the clearance of rosuvastatin in sandwich culture of primary rat hepatocytes', *Toxicol In Vitro*, 24(2), pp. 605-10.

Jenkinson, S. E., Chung, G. W., van Loon, E., Bakar, N. S., Dalzell, A. M. and Brown, C. D. A. (2012) 'The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule', *Pflügers Archiv - European Journal of Physiology*, 464(6), pp. 601-611.

Jones, P. H. and Davidson, M. H. (2005) 'Reporting rate of rhabdomyolysis with fenofibrate + statin versus gemfibrozil + any statin', *Am J Cardiol*, 95(1), pp. 120-2.

Joy, T.R. and Hegele, R.A. (2009) 'Narrative review: statin-related myopathy', *Ann Intern Med*, 150(12), pp. 858-68.

Juel, C. (1991) 'Muscle lactate transport studied in sarcolemmal giant vesicles ', *Biochim Biophys Acta*, 1065, pp. 15-20.

Juel, C. (1995) 'Regulation of cellular pH in skeletal muscle fiber types, studied with sarcolemmal giant vesicles obtained from rat muscles', *Biochim Biophys Acta*, 1265(2-3), pp. 127-132.

Juel, C. and Halestrap, A. P. (1999) 'Lactate transport in skeletal muscle — role and regulation of the monocarboxylate transporter', *The Journal of Physiology*, 517(3), pp. 633-642.

Kajinami, K., Brousseau, M. E., Ordovas, J. M. and Schaefer, E. J. (2004) 'Interactions between common genetic polymorphisms in ABCG5/G8 and CYP7A1 on LDL cholesterol-lowering response to atorvastatin', *Atherosclerosis*, 175(2), pp. 287-93.

Kajinami, K., Brousseau, M.E., Ordovas, J.M. and Schaefer, E.J. (2004b) 'Polymorphisms in the multidrug resistance-1 (MDR1) gene influence the response to atorvastatin treatment in a gender-specific manner', *Am J Cardiol*, 93(8), pp. 1046-50.

Kashani, A., Phillips, C. O., Foody, J. M., Wang, Y., Mangalmurti, S., Ko, D. T. and Krumholz, H. M. (2006) 'Risks associated with statin therapy: a systematic overview of randomized clinical trials', *Circulation*, 114(25), pp. 2788-97.

Kasten, F. H. (1999) *Introduction to fluorescent probes: Properties, history and applications*. San Diego: Academic Press.

Kaufmann, P., Torok, M., Zahno, A., Waldhauser, K. M., Brecht, K. and Krahenbuhl, S. (2006) 'Toxicity of statins on rat skeletal muscle mitochondria', *Cell Mol Life Sci*, 63(19-20), pp. 2415-25.

Keskitalo, J.E., Kurkinen, K.J., Neuvoneni, P.J. and Niemi, M. (2008) 'ABCB1 haplotypes differentially affect the pharmacokinetics of the acid and lactone forms of simvastatin and atorvastatin', *Clin Pharmacol Ther*, 84(4), pp. 457-61.

Keskitalo, J. E., Zolk, O., Fromm, M. F., Kurkinen, K. J., Neuvonen, P. J. and Niemi, M. (2009) 'ABCG2 polymorphism markedly affects the pharmacokinetics of atorvastatin and rosuvastatin', *Clin Pharmacol Ther*, 86, pp. 197-203.

Kim, R. B., Wandel, C., Leake, B., Cvetkovic, M., Fromm, M. F., Dempsey, P. J., Roden, M. M., Belas, F., Chaudhary, A. K., Roden, D. M., Wood, A. J. and Wilkinson, G. R. (1999) 'Interrelationship between substrates and inhibitors of human CYP3A and P-glycoprotein', *Pharm Res*, 16(3), pp. 408-14.

Kitamura, S., Maeda, K., Wang, Y. and Sugiyama, Y. (2008) 'Involvement of multiple transporters in the hepatobiliary transport of rosuvastatin', *Drug Metab Dispos*, 36(10), pp. 2014-23.

Knauer, M. J., Urquhart, B. L., Meyer zu Schwabedissen, H. E., Schwarz, U. I., Lemke, C. J., Leake, B. F., Kim, R. B. and Tirona, R. G. (2010) 'Human skeletal muscle drug transporters determine local exposure and toxicity of statins', *Circ Res*, 106(2), pp. 297-306.

Kobayashi, M., Itagaki, S., Hirano, T. and Iseki, K. (2004) 'Mechanism of L-lactic Acid Transport in L6 Skeletal Muscle Cells', *Drug Metab Pharmacokin*, 19(5), pp. 363-368.

Kobayashi, M., Otsuka, Y., Itagaki, S., Hirano, T. and Iseki, K. (2006) 'Inhibitory effects of statins on human monocarboxylate transporter 4', *Int J Pharm*, 317(1), pp. 19-25.

Konig, J., Rost, D., Cui, Y. and Keppler, D. (1999) 'Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane', *Hepatology*, 29(4), pp. 1156-63.

Konig, J., Seithel, A., Gradhand, U. and Fromm, M. F. (2006) 'Pharmacogenomics of human OATP transporters', *Naunyn Schmiedebergs Arch Pharmacol*, 372(6), pp. 432-43.

Kwak, H. B., Thalacker-Mercer, A., Anderson, E. J., Lin, C. T., Kane, D. A., Lee, N. S., Cortright, R. N., Bamman, M. M. and Neufer, P. D. (2012) 'Simvastatin impairs ADP-stimulated respiration and increases mitochondrial oxidative stress in primary human skeletal myotubes.', *Free Radic Biol Med*, 52(1), pp. 198-207.

Lancaster, C. S., Bruun, G. H., Peer, C. J., Mikkelsen, T. S., Corydon, T. J., Gibson, A. A., Hu, S., Orwick, S. J., Mathijssen, R. H., Figg, W. D., Baker, S. D. and Sparreboom, A. (2012) 'OATP1B1 polymorphism as a determinant of erythromycin disposition', *Clin Pharmacol Ther*, 92(5), pp. 642-50.

LaRosa, J. C., Grundy, S. M., Waters, D. D., Shear, C., Barter, P., Fruchart, J. C., Gotto, A. M., Greten, H., Kastelein, J. J., Shepherd, J. and Wenger, N. K. (2005) 'Intensive lipid lowering with atorvastatin in patients with stable coronary disease', *N Engl J Med*, 352(14), pp. 1425-1435.

Larsen, S., Stride, N., Hey-Mogensen, M., Hansen, C. N., Bang, L. E., Bundgaard, H., Nielsen, L. B., Helge, J. W. and Dela, F. (2013) 'Simvastatin effects on skeletal muscle: Relation to decreased mitochondrial function and glucose intolerance', *Journal of the American College of Cardiology*, 61(1), pp. 44-53.

Law, M. R., Wald, N. J. and Rudnicka, A. R. (2003) 'Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis', *Bmj*, 326(7404), p. 1423.

Lazar, L.D., Pletcher, M.J., Coxson, P.G., Bibbins-Domingo, K. and Goldman, L. (2011) 'Cost-effectiveness of statin therapy for primary prevention in a low-cost statin era', *Circulation*, 124(2), pp. 146-53.

Lee, E., Ryan, S., Birmingham, B., Zalikowski, J., March, R., Ambrose, H., Moore, R., Lee, C., Chen, Y. and Schneck, D. (2005) 'Rosuvastatin pharmacokinetics and pharmacogenetics in white and Asian subjects residing in the same environment', *Clin Pharmacol Ther*, 78(4), pp. 330-41.

Leier, I., Hummel-Eisenbeiss, J., Cui, Y. and Keppler, D. (2000) 'ATP-dependent para-aminohippurate transport by apical multidrug resistance protein MRP2', *Kidney Int*, 57(4), pp. 1636-42.

Lennernas, H. and Fager, G. (1997) 'Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences', *Clin Pharmacokinet*, 32(5), pp. 403-25.

Lepper, E. R., Nooter, K., Verweij, J., Acharya, M. R., Figg, W. D. and Sparreboom, A. (2005) 'Mechanisms of resistance to anticancer drugs: the role of the polymorphic ABC transporters ABCB1 and ABCG2', *Pharmacogenomics*, 6(2), pp. 115-38.

Leslie, E. M., Deeley, R. G. and Cole, S. P. (2001) 'Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters', *Toxicology*, 167(1), pp. 3-23.

Leslie, E.M., Deeley, R.G. and Cole, S.P. (2005) 'Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense', *Toxicol Appl Pharmacol*, 204(3), pp. 216-37.

Lewis, C.M. and Knight, J. (2012) 'Introduction to genetic association studies', *Cold Spring Harb Protoc*, 2012(3), pp. 297-306.

Li, Y., Gerbod-Giannone, M. C., Seitz, H., Cui, D., Thorp, E., Tall, A. R., Matsushima, G. K. and Tabas, I. (2006) 'Cholesterol-induced apoptotic macrophages elicit an inflammatory response in phagocytes, which is partially attenuated by the Mer receptor', *J Biol Chem*, 281(10), pp. 6707-17.

Liao, J. K. (2002) 'Beyond lipid lowering: the role of statins in vascular protection', *Int J Cardiol*, 86(1), pp. 5-18.

Lima, A., Bernardes, M., Azevedo, R., Monteiro, J., Sousa, H., Medeiros, R. and Seabra, V. (2014) 'SLC19A1, SLC46A1 and SLCO1B1 polymorphisms as predictors of methotrexate-related toxicity in Portuguese rheumatoid arthritis patients', *Toxicol Sci*, 142(1), pp. 196-209.

Lin, Z. and Altman, R. B. (2004) 'Finding haplotype tagging SNPs by use of principal components analysis', *Am J Hum Genet*, 75(5), pp. 850-61.

Link, E., Parish, S., Armitage, J., Bowman, L., Heath, S., Matsuda, F., Gut, I., Lathrop, M. and Collins, R. (2008) 'SLCO1B1 variants and statin-induced myopathy--a genomewide study', *N Engl J Med*, 359(8), pp. 789-99.

Liu, H. J., Yu, C. P., Hseih, Y. W., Tsai, S. Y. and Hou, Y. C. (2013) 'Inhibition of monocarboxylate transporter-mediated absorption of valproic acid by Gegen-Qinlian-Tang.', *Am J Chin Med*, 41(2), pp. 369-378.

Livak, K.J. & Schmittgen, T.D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) Method', *Methods* 25, 402-8.

Lopez-Fanjul, C., Fernandez, A. and Toro, M.A. (1999) 'The role of epistasis in the increase in the additive genetic variance after population bottlenecks', *Genetics Research*, 73(01), pp. 45-59.

Luzum, J.A., Kitzmiller, J.P., Isackson, P.J., Ma, C., Medina, M.W., Dauki, A.M., Mikulik, E.B., Ochs-Balcom, H.M. and Vladutiu, G.D. (2015) 'GATM polymorphism associated with the risk for statin-induced myopathy does not replicate in case-control analysis of 715 dyslipidemic individuals', *Cell Metab*, 21(4), pp. 622-7.

Lv, H. L., Jin, D. M., Liu, M., Liu, Y. M., Wang, J. F. and Geng, D. F. (2014) 'Long-term efficacy and safety of statin treatment beyond six years: a meta-analysis of randomized controlled trials with extended follow-up', *Pharmacol Res*, 81, pp. 64-73.

Lynch, M. and Wash, B. (1998) *Genetics and Analysis of Quantitative Traits*. Sunderland, MA: Sinauer Associates.

Macedo, A. F., Douglas, I., Smeeth, L., Forbes, H. and Ebrahim, S. (2014a) 'Statins and the risk of type 2 diabetes mellitus: cohort study using the UK clinical practice research datalink', *BMC Cardiovasc Disord*, 14, p. 85.

Macedo, A. F., Taylor, F. C., Casas, J. P., Adler, A., Prieto-Merino, D. and Ebrahim, S. (2014b) 'Unintended effects of statins from observational studies in the general population: systematic review and meta-analysis', *BMC Med*, 12, p. 51.

Maggo, S. D., Kennedy, M. A. and Clark, D. W. (2011) 'Clinical implications of pharmacogenetic variation on the effects of statins', *Drug Saf*, 34(1), pp. 1-19.

Mammen, A. L., Gaudet, D., Brisson, D., Christopher-Stine, L., Lloyd, T. E., Leffell, M. S. and Zachary, A. A. (2012) 'Increased frequency of DRB1*11:01 in anti-

hydroxymethylglutaryl-coenzyme A reductase-associated autoimmune myopathy', *Arthritis Care Res (Hoboken)*, 64(8), pp. 1233-7.

Mangravite, L. M., Engelhardt, B. E., Medina, M. W., Smith, J. D., Brown, C. D., Chasman, D. I., Mecham, B. H., Howie, B., Shim, H., Naidoo, D., Feng, Q., Rieder, M. J., Chen, Y. D., Rotter, J. I., Ridker, P. M., Hopewell, J. C., Parish, S., Armitage, J., Collins, R., Wilke, R. A., Nickerson, D. A., Stephens, M. and Krauss, R. M. (2013) 'A statin-dependent QTL for GATM expression is associated with statin-induced myopathy', *Nature*, 502(7471), pp. 377-80.

Manning Fox, J. E., Meredith, D. and Halestrap, A. P. (2000) 'Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle', *J Physiol*, 529 Pt 2, pp. 285-93.

Marciante, K. D., Durda, J. P., Heckbert, S. R., Lumley, T., Rice, K., McKnight, B., Totah, R. A., Tamraz, B., Kroetz, D. L., Fukushima, H., Kaspera, R., Bis, J. C., Glazer, N. L., Li, G., Austin, T. R., Taylor, K. D., Rotter, J. I., Jaquish, C. E., Kwok, P. Y., Tracy, R. P. and Psaty, B. M. (2011) 'Cerivastatin, genetic variants, and the risk of rhabdomyolysis', *Pharmacogenet Genomics*, 21(5), pp. 280-8.

Marcoff, L. and Thompson, P. D. (2007) 'The role of coenzyme Q10 in statin-associated myopathy: a systematic review', *J Am Coll Cardiol*, 49(23), pp. 2231-7.

Maron, D. J., Fazio, S. and Linton, M. F. (2000) 'Current perspectives on statins', *Circulation*, 101(2), pp. 207-13.

Maxa, J. L., Melton, L. B., Ogu, C. C., Sills, M. N. and Limanni, A. (2002) 'Rhabdomyolysis after concomitant use of cyclosporine, simvastatin, gemfibrozil, and itraconazole', *Ann Pharmacother*, 36(5), pp. 820-3.

McClure, D.L., Valuck, R.J., Glanz, M. and Hokanson, J.E. (2007) 'Systematic review and meta-analysis of clinically relevant adverse events from HMG CoA reductase inhibitor trials worldwide from 1982 to present', *Pharmacoepidemiol Drug Saf*, 16(2), pp. 132-43.

McCullagh, K. J., Poole, R. C., Halestrap, A. P., O'Brien, M. and Bonen, A. (1996) 'Role of the lactate transporter (MCT1) in skeletal muscles', *Am J Physiol* 271(1 Pt 1), pp. E143-E150.

McDermott, J. C. and Bonen, A. (1993) 'Endurance training increases skeletal muscle lactate transport', *Acta Physiol Scand*, 147, pp. 323-327.

McDermott, J. C. and Bonen, A. (1993) 'Lactate transport by skeletal muscle sarcolemmal vesicles ', *Mol Cell Biochem*, 122, pp. 113-121.

McTaggart, F., Buckett, L., Davidson, R., Holdgate, G., McCormick, A., Schneck, D., Smith, G. and Warwick, M. (2001) 'Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor', *Am J Cardiol*, 87(5a), pp. 28b-32b.

McTavish, D. and Sorkin, E. M. (1991) 'Pravastatin. A review of its pharmacological properties and therapeutic potential in hypercholesterolaemia', *Drugs*, 42(1), pp. 65-89.

Men, T., Zhang, X., Yang, J., Shen, B., Li, X., Chen, D. and Wang, J. (2014) 'The rs1050450 C > T polymorphism of GPX1 is associated with the risk of bladder but not prostate cancer: evidence from a meta-analysis', *Tumour Biol*, 35(1), pp. 269-75.

Miles, L., Miles, M. V., Tang, P. H., Horn, P. S., Wong, B. L., DeGrauw, T. J., Morehart, P. J. and Bove, K. E. (2005) 'Muscle coenzyme Q: a potential test for mitochondrial activity and redox status', *Pediatr Neurol*, 32(5), pp. 318-24.

Monteiro, M. B., Patente, T. A., Mohammedi, K., Queiroz, M. S., Azevedo, M. J., Canani, L. H., Parisi, M. C., Marre, M., Velho, G. and Correa-Giannella, M. L. (2013) 'Sex-specific associations of variants in regulatory regions of NADPH oxidase-2 (CYBB) and glutathione peroxidase 4 (GPX4) genes with kidney disease in type 1 diabetes', *Free Radic Res*, 47(10), pp. 804-10.

Morgan, S. A., Watson, J. V., Twentyman, P. R. and Smith, P. J. (1989) 'Flow cytometric analysis of Hoechst 33342 uptake as an indicator of multi-drug resistance in human lung cancer', *Br J Cancer*, 60(3), pp. 282-7.

Morse, B. L., Vijay, N. and Morris, M. E. (2014) 'Mechanistic modeling of monocarboxylate transporter-mediated toxicokinetic/toxicodynamic interactions between γ-hydroxybutyrate and L-lactate.', *AAPS J*, 16(4), pp. 756-770.

Mosshammer, D., Schaeffeler, E., Schwab, M. and Morike, K. (2014) 'Mechanisms and assessment of statin-related muscular adverse effects', *Br J Clin Pharmacol*, 78(3), pp. 454-66.

Mueller, C. F., Widder, J. D., McNally, J. S., McCann, L., Jones, D. P. and Harrison, D. G. (2005) 'The role of the multidrug resistance protein-1 in modulation of endothelial cell oxidative stress', *Circ Res*, 97(7), pp. 637-44.

Muldoon, M. F., Barger, S. D., Ryan, C. M., Flory, J. D., Lehoczky, J. P., Matthews, K. A. and Manuck, S. B. (2000) 'Effects of lovastatin on cognitive function and psychological well-being', *Am J Med*, 108(7), pp. 538-46.

Muller, H., Klinkhammer, W., Globisch, C., Kassack, M. U., Pajeva, I. K. and Wiese, M. (2007) 'New functional assay of P-glycoprotein activity using Hoechst 33342', *Bioorg Med Chem*, 15(23), pp. 7470-9.

Nagasawa, K., Nagai, K., Ishimoto, A. and Fujimoto, S. (2003) 'Transport mechanism for lovastatin acid in bovine kidney NBL-1 cells: kinetic evidences imply involvement of monocarboxylate transporter 4', *Int J Pharm*, 262(1-2), pp. 63-73.

Nakahara, K., Kuriyama, M., Sonoda, Y., Yoshidome, H., Nakagawa, H., Fujiyama, J., Higuchi, I. and Osame, M. (1998) 'Myopathy induced by HMG-CoA reductase inhibitors in rabbits: a pathological, electrophysiological, and biochemical study', *Toxicol Appl Pharmacol*, 152(1), pp. 99-106.

Nathan, C. F. (1987) 'Secretory products of macrophages', *J Clin Invest*, 79(2), pp. 319-26.

National Clinical Guideline Centre (NCGC). (2014) 'National Institute for Health and Clinical Excellence: Guidance', in *Lipid Modification: Cardiovascular Risk Assessment and the Modification of Blood Lipids for the Primary and Secondary Prevention of Cardiovascular Disease*. London: National Institute for Health and Care Excellence (UK). Copyright (c) National Clinical Guideline Centre, 2014.

Navarese, E. P., Buffon, A., Andreotti, F., Kozinski, M., Welton, N., Fabiszak, T., Caputo, S., Grzesk, G., Kubica, A., Swiatkiewicz, I., Sukiennik, A., Kelm, M., De Servi, S. and Kubica, J. (2013) 'Meta-analysis of impact of different types and doses of statins on new-onset diabetes mellitus', *Am J Cardiol*, 111(8), pp. 1123-30.

Needham, M. and Mastaglia, F. L. (2014) 'Statin myotoxicity: a review of genetic susceptibility factors', *Neuromuscul Disord*, 24(1), pp. 4-15.

Neuvonen, P. J., Niemi, M. and Backman, J. T. (2006) 'Drug interactions with lipid-lowering drugs: mechanisms and clinical relevance', *Clin Pharmacol Ther*, 80(6), pp. 565-81.

Newman, C., Tsai, J., Szarek, M., Luo, D. and Gibson, E. (2006) 'Comparative safety of atorvastatin 80 mg versus 10 mg derived from analysis of 49 completed trials in 14,236 patients', *Am J Cardiol*, 97(1), pp. 61-7.

Nicolas, P., Sun, F. and Li, L. M. (2006) 'A model-based approach to selection of tag SNPs', *BMC Bioinformatics*, 7, p. 303.

Niemi, M. (2010) 'Transporter pharmacogenetics and statin toxicity', *Clin Pharmacol Ther*, 87(1), pp. 130-3.

Niemi, M., Pasanen, M. K. and Neuvonen, P. J. (2006) 'SLCO1B1 polymorphism and sex affect the pharmacokinetics of pravastatin but not fluvastatin', *Clin Pharmacol Ther*, 80(4), pp. 356-66.

Nies, A. T., Niemi, M., Burk, O., Winter, S., Zanger, U. M., Stieger, B., Schwab, M. and Schaeffeler, E. (2013) 'Genetics is a major determinant of expression of the human hepatic uptake transporter OATP1B1, but not of OATP1B3 and OATP2B1', *Genome Med*, 5(1), p. 1.

Norata, G. D., Tibolla, G. and Catapano, A. L. (2014) 'Statins and skeletal muscles toxicity: from clinical trials to everyday practice', *Pharmacol Res*, 88, pp. 107-13

Nordestgaard, B. G., Chapman, M. J., Humphries, S. E., Ginsberg, H. N., Masana, L., Descamps, O. S., Wiklund, O., Hegele, R. A., Raal, F. J., Defesche, J. C., Wiegman, A., Santos, R. D., Watts, G. F., Parhofer, K. G., Hovingh, G. K., Kovanen, P. T., Boileau, C., Averna, M., Boren, J., Bruckert, E., Catapano, A. L., Kuivenhoven, J. A., Pajukanta, P., Ray, K., Stalenhoef, A. F., Stroes, E., Taskinen, M. R. and Tybjaerg-Hansen, A. (2013) 'Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society', *Eur Heart J*, 34(45), pp. 3478-90a.

Obayashi, H., Nezu, Y., Yokota, H., Kiyosawa, N., Mori, K., Maeda, N., Tani, Y., Manabe, S. and Sanbuissho, A. (2011) 'Cerivastatin induces type-I fiber-, not type-II fiber-, predominant muscular toxicity in the young male F344 rats', *J Toxicol Sci*, 36(4), pp. 445-52.

Oh, J., Ban, M. R., Miskie, B. A., Pollex, R. L. and Hegele, R. A. (2007) 'Genetic determinants of statin intolerance', *Lipids Health Dis*, 6, p. 7.

Omar, M. A. and Wilson, J. P. (2002) 'FDA adverse event reports on statin-associated rhabdomyolysis', *Ann Pharmacother*, 36(2), pp. 288-95.

O'Meara, H., Carr, D.F., Evely, J., Hobbs, M., McCann, G., van Staa, T. and Pirmohamed, M. (2014) 'Electronic health records for biological sample collection: feasibility study of statin-induced myopathy using the Clinical Practice Research Datalink', *Br J Clin Pharmacol*, 77(5), pp. 831-8.

Parker, B. A., Capizzi, J. A., Grimaldi, A. S., Clarkson, P. M., Cole, S. M., Keadle, J., Chipkin, S., Pescatello, L. S., Simpson, K., White, C. M. and Thompson, P. D. (2013) 'Effect of statins on skeletal muscle function', *Circulation*, 127(1), pp. 96-103.

Parker, B. A. and Thompson, P. D. (2012) 'Effect of Statins on Skeletal Muscle: Exercise, Myopathy, and Muscle Outcomes', *Exerc. Sport Sci. Rev*, 40(4), pp. 188-194.

Pasanen, M. K., Fredrikson, H., Neuvonen, P. J. and Niemi, M. (2007) 'Different effects of SLCO1B1 polymorphism on the pharmacokinetics of atorvastatin and rosuvastatin', *Clin Pharmacol Ther*, 82(6), pp. 726-33.

Pasanen, M. K., Neuvonen, M., Neuvonen, P. J. and Niemi, M. (2006) 'SLCO1B1 polymorphism markedly affects the pharmacokinetics of simvastatin acid', *Pharmacogenet Genomics*, 16(12), pp. 873-9.

Pasternak, R. C., Smith, S. C., Jr., Bairey-Merz, C. N., Grundy, S. M., Cleeman, J. I. and Lenfant, C. (2002) 'ACC/AHA/NHLBI Clinical Advisory on the Use and Safety of Statins', *Circulation*, 106(8), pp. 1024-8.

Pedersen, T. R., Faergeman, O., Kastelein, J. J., Olsson, A. G., Tikkanen, M. J., Holme, I., Larsen, M. L., Bendiksen, F. S., Lindahl, C., Szarek, M. and Tsai, J. (2005) 'High-dose atorvastatin vs usual-dose simvastatin for secondary prevention after myocardial infarction: the IDEAL study: a randomized controlled trial', *Jama*, 294(19), pp. 2437-45.

Pedro-Botet, J., Schaefer, E.J., Bakker-Arkema, R.G., Black, D.M., Stein, E.M., Corella, D. and Ordovas, J.M. (2001) 'Apolipoprotein E genotype affects plasma lipid response to atorvastatin in a gender specific manner', *Atherosclerosis*, 158(1), pp. 183-93.

Pfeifer, N. D., Yang, K. and Brouwer, K. L. (2013) 'Hepatic basolateral efflux contributes significantly to rosuvastatin disposition I: characterization of basolateral versus biliary clearance using a novel protocol in sandwich-cultured hepatocytes', *J Pharmacol Exp Ther*, 347(3), pp. 727-36.

Phillips, P. S., Haas, R. H., Bannykh, S., Hathaway, S., Gray, N. L., Kimura, B. J., Vladutiu, G. D. and England, J. D. (2002) 'Statin-associated myopathy with normal creatine kinase levels', *Ann Intern Med*, 137(7), pp. 581-5.

Pia Iglesias, G., Fernandez Fernandez, F. J., Ameneiros Lago, E. and Sesma Sanchez, P. (2001) '[HMG-CoA reductase inhibitors and sexual dysfunction]', *An Med Interna*, 18(3), p. 171.

Picard, M., Hepple, R. T. and Burelle, Y. (2012) 'Mitochondrial functional specialization in glycolytic and oxidative muscle fibers: tailoring the organelle for optimal function', *Am J Physiol Cell Physiol*, 302(4), pp. C629-41.

Pilegaard, H., Terzis, G., Halestrap, A. and Juel, C. (1999) 'Distribution of the lactate/H+ transporter isoforms MCT1 and MCT4 in human skeletal muscle ', *Am J Physiol Endocrinol Metab*, 276, pp. E843-E848.

Preiss, D., Seshasai, S. R., Welsh, P., Murphy, S. A., Ho, J. E., Waters, D. D., DeMicco, D. A., Barter, P., Cannon, C. P., Sabatine, M. S., Braunwald, E., Kastelein, J. J., de Lemos, J. A., Blazing, M. A., Pedersen, T. R., Tikkanen, M. J., Sattar, N. and Ray, K. K. (2011) 'Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy: a meta-analysis', *Jama*, 305(24), pp. 2556-64.

Psaty, B.M. and Vandenbroucke, J.P. (2008) 'Opportunities for enhancing the FDA guidance on pharmacovigilance', *Jama*, 300(8), pp. 952-4.

Puccetti, L., Ciani, F. and Auteri, A. (2010) 'Genetic involvement in statins induced myopathy. Preliminary data from an observational case-control study.', *Atherosclerosis*, 211(1), pp. 28-29.

Rajpathak, S. N., Kumbhani, D. J., Crandall, J., Barzilai, N., Alderman, M. and Ridker, P. M. (2009) 'Statin therapy and risk of developing type 2 diabetes: a meta-analysis', *Diabetes Care*, 32(10), pp. 1924-9.

Reiner, Z. (2014) 'Resistance and intolerance to statins', *Nutr Metab Cardiovasc Dis*, 24(10), pp. 1057-1066.

Reiner, Z. (2015) 'PCSK9 inhibitors - past, present and future', *Expert Opin Drug Metab Toxicol*, 11(10), pp. 1517-21.

Ridker, P. M., Danielson, E., Fonseca, F. A., Genest, J., Gotto, A. M., Jr., Kastelein, J. J., Koenig, W., Libby, P., Lorenzatti, A. J., MacFadyen, J. G., Nordestgaard, B. G., Shepherd, J., Willerson, J. T. and Glynn, R. J. (2008) 'Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein', *N Engl J Med*, 359(21), pp. 2195-207.

Risch, N. J. (2000) 'Searching for genetic determinants in the new millennium', *Nature*, 405(6788), pp. 847-56.

Rodrigues, A. C. (2010) 'Efflux and uptake transporters as determinants of statin response', *Expert Opin Drug Metab Toxicol*, 6(5), pp. 621-32.

Rosenson, R. S. and Bays, H. E. (2003) 'Results of two clinical trials on the safety and efficacy of pravastatin 80 and 160 mg per day', *Am J Cardiol*, 91(7), pp. 878-81.

Roses, A. D. (2000) 'Pharmacogenetics and the practice of medicine', *Nature*, 405(6788), pp. 857-65.

Roth, D. A. and Brooks, G. A. (1990) 'Lactate transport is mediated by membrane-bound carrier in rat skeletal muscle sarcolemmal vesicles ', *Arch Biochem Biophys*, 279, pp. 377-385.

Rowan, C., Brinker, A. D., Nourjah, P., Chang, J., Mosholder, A., Barrett, J. S. and Avigan, M. (2009) 'Rhabdomyolysis reports show interaction between simvastatin and CYP3A4 inhibitors', *Pharmacoepidemiol Drug Saf*, 18(4), pp. 301-9.

Sadee, W. (2013) 'Gene-gene-environment interactions between drugs, transporters, receptors, and metabolizing enzymes: Statins, SLCO1B1, and CYP3A4 as an example', *J Pharm Sci*, 102(9), pp. 2924-9.

Sahebkar, A. and Watts, G.F. (2013) 'New LDL-cholesterol lowering therapies: pharmacology, clinical trials, and relevance to acute coronary syndromes', *Clin Ther*, 35(8), pp. 1082-98.

Sasieni, P. D. (1997) 'From genotypes to genes: doubling the sample size', *Biometrics*, 53(4), pp. 1253-61.

Sattar, N., Preiss, D., Murray, H. M., Welsh, P., Buckley, B. M., de Craen, A. J., Seshasai, S. R., McMurray, J. J., Freeman, D. J., Jukema, J. W., Macfarlane, P. W., Packard, C. J., Stott, D. J., Westendorp, R. G., Shepherd, J., Davis, B. R., Pressel, S. L., Marchioli, R., Marfisi, R. M., Maggioni, A. P., Tavazzi, L., Tognoni, G., Kjekshus, J., Pedersen, T. R., Cook, T. J., Gotto, A. M., Clearfield, M. B., Downs, J. R., Nakamura, H., Ohashi, Y., Mizuno, K., Ray, K. K. and Ford, I. (2010) 'Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials', *Lancet*, 375(9716), pp. 735-42.

Sattar, N. and Taskinen, M. R. (2012) 'Statins are diabetogenic--myth or reality?', *Atheroscler Suppl*, 13(1), pp. 1-10.

Scandinavian Simvastatin Survival Study Group (1994) 'Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S)', *Lancet*, 344(8934), pp. 1383-9.

Schachter, M. (2005) 'Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update', *Fundam Clin Pharmacol*, 19(1), pp. 117-25.

Schech, S., Graham, D., Staffa, J., Andrade, S. E., La Grenade, L., Burgess, M., Blough, D., Stergachis, A., Chan, K. A., Platt, R. and Shatin, D. (2007) 'Risk factors for statin-associated rhabdomyolysis', *Pharmacoepidemiol Drug Saf*, 16(3), pp. 352-8.

Schmittgen, T.D. and Livak, K.J. (2008) 'Analyzing real-time PCR data by the comparative C_T method', *Nat Protoc*, 3(6), pp. 1101-8.

Schwab, M., Eichelbaum, M. and Fromm, M.F. (2003) 'Genetic polymorphisms of the human MDR1 drug transporter', *Annu Rev Pharmacol Toxicol*, 43, pp. 285-307.

Senior, P. A. and Bhopal, R. (1994) 'Ethnicity as a variable in epidemiological research', *Bmj*, 309(6950), pp. 327-30.

Sever, P. S., Dahlof, B., Poulter, N. R., Wedel, H., Beevers, G., Caulfield, M., Collins, R., Kjeldsen, S. E., Kristinsson, A., McInnes, G. T., Mehlsen, J., Nieminen, M., O'Brien, E. and Ostergren, J. (2003) 'Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial', *Lancet*, 361(9364), pp. 1149-58.

Shek, A. and Ferrill, M. J. (2001) 'Statin-fibrate combination therapy', *Ann Pharmacother*, 35(7-8), pp. 908-17.

Shepherd, J., Cobbe, S. M., Ford, I., Isles, C. G., Lorimer, A. R., MacFarlane, P. W., McKillop, J. H. and Packard, C. J. (1995) 'Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group', *N Engl J Med*, 333(20), pp. 1301-7.

Shitara, Y. and Sugiyama, Y. (2006) 'Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions', *Pharmacol Ther*, 112(1), pp. 71-105.

Sirtori, C. R., Mombelli, G., Triolo, M. and Laaksonen, R. (2012) 'Clinical response to statins: mechanism(s) of variable activity and adverse effects', *Ann Med*, 44(5), pp. 419-32.

Sirvent, P., Bordenave, S., Vermaelen, M., Roels, B., Vassort, G., Mercier, J., Raynaud, E. and Lacampagne, A. (2005) 'Simvastatin induces impairment in skeletal muscle while heart is protected', *Biochem Biophys Res Commun* 338(3), pp. 1426-1434.

Sirvent, P., Mercier, J. and Lacampagne, A. (2008) 'New insights into mechanisms of statin-associated myotoxicity', *Respiratory/Musculoskeletal*, 8(3), pp. 333-338.

Sissung, T. M., Baum, C. E., Kirkland, C. T., Gao, R., Gardner, E. R. and Figg, W. D. (2010) 'Pharmacogenetics of membrane transporters: an update on current approaches', *Mol Biotechnol*, 44(2), pp. 152-67.

Smilde, T. J., van Wissen, S., Wollersheim, H., Trip, M. D., Kastelein, J. J. and Stalenhoef, A. F. (2001) 'Effect of aggressive versus conventional lipid lowering on atherosclerosis progression in familial hypercholesterolaemia (ASAP): a prospective, randomised, double-blind trial', *Lancet*, 357(9256), pp. 577-81.

Smith, M. E. B., Lee, N. J., Haney, E. and Carson, S. (2009) 'Drug Class Reviews', in *Drug Class Review: HMG-CoA Reductase Inhibitors (Statins) and Fixed-dose Combination Products Containing a Statin: Final Report Update 5*. Portland, Oregon: Oregon Health & Science University.

Sparreboom, A., Gelderblom, H., Marsh, S., Ahluwalia, R., Obach, R., Principe, P., Twelves, C., Verweij, J. and McLeod, H. L. (2004) 'Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype', *Clin Pharmacol Ther*, 76(1), pp. 38-44.

Sreenarasimhaiah, J., Shiels, P. and Lisker-Melman, M. (2002) 'Multiorgan failure induced by atorvastatin', *Am J Med*, 113(4), pp. 348-9.

Stewart, A. (2013) 'SLCO1B1 Polymorphisms and Statin-Induced Myopathy', *PLoS Curr*, 5.

Stram, D. O. (2004) 'Tag SNP selection for association studies', *Genet Epidemiol*, 27(4), pp. 365-74.

Stringer, H. A., Sohi, G. K., Maguire, J. A. and Cote, H. C. (2013) 'Decreased skeletal muscle mitochondrial DNA in patients with statin-induced myopathy.', *J Neurol Sci*, 325(1-2), pp. 142-147.

Szadkowska, I., Stanczyk, A., Aronow, W. S., Kowalski, J., Pawlicki, L., Ahmed, A. and Banach, M. (2010) 'Statin therapy in the elderly: a review', *Arch Gerontol Geriatr*, 50(1), pp. 114-8.

Taha, D. A., De Moor, C. H., Barrett, D. A. and Gershkovich, P. (2014) 'Translational insight into statin-induced muscle toxicity: from cell culture to clinical studies', *Transl Res*, 164(2), pp. 85-109.

Takeda, M., Noshiro, R., Onozato, M.L., Tojo, A., Hasannejad, H., Huang, X.L., Narikawa, S. and Endou, H. (2004) 'Evidence for a role of human organic anion transporters in the muscular side effects of HMG-CoA reductase inhibitors', *Eur J Pharmacol*, 483(2-3), pp. 133-8.

Talbert, R. L. (2006) 'Safety issues with statin therapy', *J Am Pharm Assoc (2003)*, 46(4), pp. 479-88; quiz 488-90.

Tay, S. K., Dimauro, S., Pang, A. Y., Lai, P. S. and Yap, H. K. (2008) 'Myotoxicity of lipid-lowering agents in a teenager with MELAS mutation', *Pediatr Neurol*, 39(6), pp. 426-8.

Thomas, C., Bishop, D. J., Lambert, K., Mercier, J. and Brooks, G. A. (2012) 'Effects of acute and chronic exercise on sarcolemmal MCT1 and MCT4 contents in human skeletal muscles: current status', *Am J Physiol Regul Integr Comp Physiol*, 302(1), pp. R1-14.

Thomas, J. E., Lee, N. and Thompson, P. D. (2007) 'Statins provoking MELAS syndrome. A case report', *Eur Neurol*, 57(4), pp. 232-5.

Thompson, P. D., Clarkson, P. and Karas, R. H. (2003) 'Statin-associated myopathy', *Jama*, 289(13), pp. 1681-90.

Thompson, P. D., Clarkson, P. M. and Rosenson, R. S. (2006) 'An Assessment of Statin Safety by Muscle Experts', *The American Journal of Cardiology*, 97(8A), pp. 69C-76C.

Thompson, P. D., Clarkson, P. M. and Rosenson, R. S. (2006) 'An assessment of statin safety by muscle experts', *Am J Cardiol*, 97(8a), pp. 69c-76c.

Thompson, P. D., Zmuda, J. M., Domalik, L. J., Zimet, R. J., Staggers, J. and Guyton, J. R. (1997) 'Lovastatin increase exercise-induced skeletal muscle injury.', *Metabolism*, 46(10), pp. 1206-1210.

Townsend, N., Wickramasinghe, K., Bhatnagar, P., Smolina, K., Nichols, M., Leal, J., Luengo-Fernandez, R. and Rayner, M. (2012) *Coronary heart disease statistics 2012 edition*. London, UK: British Heart Foundation.

Troseid, M., Henriksen, O. A. and Lindal, S. (2005) 'Statin-associated myopathy with normal creatine kinase levels. Case report from a Norwegian family', *Apmis*, 113(9), pp. 635-7.

Tsamandouras, N., Dickinson, G., Guo, Y., Hall, S., Rostami-Hodjegan, A., Galetin, A. and Aarons, L. (2014) 'Identification of the effect of multiple polymorphisms on the pharmacokinetics of simvastatin and simvastatin acid using a population-modeling approach', *Clin Pharmacol Ther*, 96(1), pp. 90-100.

van de Woestijne, A. P., van der Graaf, Y., Westerink, J., Nathoe, H. M. and Visseren, F. L. (2015) 'Effect of statin therapy on incident type 2 diabetes mellitus in patients with clinically manifest vascular disease', *Am J Cardiol*, 115(4), pp. 441-6.

Vaughan, C. J., Gotto, A. M., Jr. and Basson, C. T. (2000) 'The evolving role of statins in the management of atherosclerosis', *J Am Coll Cardiol*, 35(1), pp. 1-10.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., et al. (2001) 'The sequence of the human genome', *Science*, 291(5507), pp. 1304-51.

Vladutiu, G. D., Simmons, Z., Isackson, P. J., Tarnopolsky, M., Peltier, W. L., Barboi, A. C., Sripathi, N., Wortmann, R. L. and Phillips, P. S. (2006) 'Genetic risk factors

associated with lipid-lowering drug-induced myopathies', *Muscle Nerve*, 34(2), pp. 153-62.

Voora, D., Shah, S. H., Spasojevic, I., Ali, S., Reed, C. R., Salisbury, B. A. and Ginsburg, G. S. (2009) 'The SLCO1B1*5 genetic variant is associated with statin-induced side effects', *J Am Coll Cardiol*, 54(17), pp. 1609-16.

Wang, J., Caballero, A., Keightley, P.D. and Hill, W.G. (1998) 'Bottleneck effect on genetic variance. A theoretical investigation of the role of dominance', *Genetics*, 150(1), pp. 435-47.

Wang, Q., Lu, Y., Yuan, M., Darling, I. M., Repasky, E. A. and Morris, M. E. (2006) 'Characterization of monocarboxylate transport in human kidney HK-2 cells', *Mol Pharm*, 3(6), pp. 675-85.

Waters, D. D. (2005) 'Safety of high-dose atorvastatin therapy', *Am J Cardiol*, 96(5a), pp. 69f-75f.

Weir, B. S. (1996) *Genetic data analysis II: Methods for discrete population genetic data.* Sudnerland, MA: Sinauer Associates, Inc.

Wilke, R. A., Ramsey, L. B., Johnson, S. G., Maxwell, W. D., McLeod, H. L., Voora, D., Krauss, R. M., Roden, D. M., Feng, Q., Cooper-Dehoff, R. M., Gong, L., Klein, T. E., Wadelius, M. and Niemi, M. (2012) 'The clinical pharmacogenomics implementation consortium: CPIC guideline for SLCO1B1 and simvastatin-induced myopathy', *Clin Pharmacol Ther*, 92(1), pp. 112-7.

Willis, R. A., Folkers, K., Tucker, J. L., Ye, C. Q., Xia, L. J. and Tamagawa, H. (1990) 'Lovastatin decreases coenzyme Q levels in rats', *Proc Natl Acad Sci U S A*, 87(22), pp. 8928-30.

Wilson, M. C., Jackson, V. N., Heddle, C., Price, N. T., Pilegaard, H., Juel, C., Bonen, A., Montgomery, I., Hutter, O. F. and Halestrap, A. P. (1998) 'Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3', *J Biol Chem*, 273(26), pp. 15920-6.

World Health Organisation (2008) *The global burden of disease: 2004 update*. Available at: http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/ (Accessed: 15 July 2011).

Xiao, J. J., Foraker, A. B., Swaan, P. W., Liu, S., Huang, Y., Dai, Z., Chen, J., Sadee, W., Byrd, J., Marcucci, G. and Chan, K. K. (2005) 'Efflux of depsipeptide FK228 (FR901228, NSC-630176) is mediated by P-glycoprotein and multidrug resistance-associated protein 1', *J Pharmacol Exp Ther*, 313(1), pp. 268-76.

Xie, H. G., Kim, R. B., Wood, A. J. and Stein, C. M. (2001) 'Molecular basis of ethnic differences in drug disposition and response', *Annu Rev Pharmacol Toxicol*, 41, pp. 815-50.

Yaffe, D. (1968) 'Retention of differentiation potentialities during prolonged cultivation of myogenic cells', *Proc Natl Acad Sci U S A*, 61(2), pp. 477-83.

Zaharan, N. L., Williams, D. and Bennett, K. (2013) 'Statins and risk of treated incident diabetes in a primary care population', *Br J Clin Pharmacol*, 75(4), pp. 1118-24.

Zhang, K., Calabrese, P., Nordborg, M. and Sun, F. (2002) 'Haplotype block structure and its applications to association studies: power and study designs', *Am J Hum Genet*, 71(6), pp. 1386-94.

Zhang, K., Qin, Z. S., Liu, J. S., Chen, T., Waterman, M. S. and Sun, F. (2004) 'Haplotype block partitioning and tag SNP selection using genotype data and their applications to association studies', *Genome Res*, 14(5), pp. 908-16.

Zhang, K. and Sun, F. (2007) 'Tag SNP Selection and Its Application in Association Studies ', in Deng, H.-W., Shen, H., Liu, Y.-J. and Hu, H. (eds.) *Current Topics in Human Genetics: Studies in Complex Diseases*. Singapore: World Scientific Publishing Co. Pte. Ltd.

Zheng, G., Yang, Y., Zhu, X. and Elston, R. C. (2012) *Analysis of Genetic Association Studies*. New York, USA: Springer.

Zhou, Q., Ruan, Z. R., Yuan, H., Xu, D. H. and Zeng, S. (2013) 'ABCB1 gene polymorphisms, ABCB1 haplotypes and ABCG2 c.421c > A are determinants of inter-subject variability in rosuvastatin pharmacokinetics', *Pharmazie*, 68(2), pp. 129-34.

Ziegler, K. and Stunkel, W. (1992) 'Tissue-selective action of pravastatin due to hepatocellular uptake via a sodium-independent bile acid transporter', *Biochim Biophys Acta*, 1139(3), pp. 203-9.