Title:

Prevalence of SLCO1B1 single nucleotide variations and their association with hypercholesterolemia in hypercholesterolemic patients in Gauteng, South Africa.

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Abstract

Background and aim: Statins, the standard treatment for hypercholesterolemia, among the most widely prescribed, have been associated with side effects, including statin intolerance. The aim of this study was to determine the prevalence of *SLCO1B1* single nucleotide variations (SNVs) and possible associations between *SLCO1B1* SNVs, statin intolerance and creatine kinase (CK) in hypercholesterolemic patients on statin therapy.

Methodology: 181 healthy controls and 100 hypercholesterolemic patients receiving either simvastatin (71%) or atorvastatin (29%) were recruited. Statin intolerance risk was calculated using the quantitative questionnaire based on the American College of Cardiology's Statin Intolerance Application. Using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP), the presence of *SLCO1B1* SNVs (*rs4149056, rs2306283* and *rs4363657*) was identified, while enzyme-linked immunosorbent assay (ELISA) was used to quantify serum creatine kinase (CK) levels.

Results: Of the 100 hypercholesterolemic patients, 15% were at high risk, 49% at moderate risk and 36% at low risk for statin intolerance. The prevalence of the *rs4149056* variant was 16% for the control group and 20% for the treatment group. (OR=1.324; 95% CI=0.8430 to 2.078; *p*=0.2405). The *rs2306283* variant was present in 31.5% of the control group compared to only 10.5% in the treatment group (OR=0.2552 95% CI=0.1542 to 0.4223, *p*< 0.0001), while the prevalence of the *rs4363657* variant was similar in each group. (OR=1.345, 95% CI=0.8492 to 2.129, *p*=0.2380).

A comparison of genotype frequencies based on calculated statin intolerance risk showed no significant association between any of the SNVs, (*rs4149056*, OR=0.7857, 95% CI=0.2115 to 2.919, relative risk (RR)=0.8800, 95% CI=0.4433 to 1.747, p=0.7496, *rs2306283*, OR=0.4911, 95% CI=0.1234 to 1.954, RR=0.9659, 95% CI= 0.4888 to 1.909, p=0.4877 and *rs4363657*, OR=0.9375, 95% CI=0.2634 to 3.3337, RR=0.6984, 95% CI=0.3609 to 1.352, *p*=1.0000). CK levels in patients on simvastatin were significantly higher compared to patients on atorvastatin (*p*=0.0418).

Conclusion: The prevalence of the *SLCO1B1* SNVs in this population is a novel finding. No association between the presence of any one of the SNVs and the statin intolerance severity risk score or CK elevation was found.

Keywords:

SLCO1B1, Statins, Atorvastatin, Simvastatin, Hypercholesterolemia, Statin Intolerance

Introduction

The treatment of hypercholesterolemia has become one of the most important clinical issues in modern medicine; due to the high burden elevated low-density lipoprotein (LDL) levels pose on cardiovascular risk. Increased LDL levels increase the risk for cardiovascular disease (CVD) substantially and extensive research shows that reducing LDL decreases this risk significantly both in patients presenting with hypercholesterolemia and in those with comparatively normal levels of LDL. (De Backer and others 2003; Martin and others 2012)

Bile-acid binding resins, fibrates, nicotinic acid, cholesterol absorption inhibitors, and hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors (Bonfim and others 2015) are currently used to lower LDL levels. HMG-CoA reductase inhibitors, commonly known as statins, are the most well-known and universally prescribed lipid-lowering therapies. (Hanai and others 2007; Schachter 2005)

At tertiary institutions in South Africa, simvastatin, and atorvastatin are most frequently prescribed, in combination with dietary and lifestyle changes in order to ensure effective lowering of LDL and elevation of high-density lipoprotein (HDL). The benefits of statins outweigh their risks. (Shah and Goldfine 2012) Statins are linked to side effects, in particular, statin intolerance. (Brunham and others 2012a; Kellick and others 2014a) Various associations and authorities differ in their definition of statin intolerance. The National Lipid Association (NLA), International Lipid Expert Panel (ILEP) and the Canadian Consensus Working Group (CCWG) agree that statin intolerance is a clinical syndrome, which manifests as the inability to tolerate at least two statins, (one of which is at its lowest daily dose), due to symptoms and signs related to statin treatment, e.g., increase in laboratory markers and / or myopathy. These usually resolve upon withdrawal of statin therapy. (Banach and others 2015; Jacobson and others 2014; Mancini and others 2016; Toth and others 2018)

Various factors, such as age, gender, drug-drug interactions, and genetic variations in genes encoding for drug transporters are implicated in the pathogenesis of statin

induced myopathy. (Petrkova and others 2018) Genetic variations, such as single nucleotide variations (SNVs), may affect the pharmacokinetics and hence plasma concentrations and total exposure to a drug. (Alwi 2005; Cuevas and others 2016; Thompson and others 2009) Muscle related adverse events associated with statins may be due to inadequate metabolism and clearance of statins resulting in increased plasma concentrations and / or prolonged exposure, leading to toxicity. It is hypothesized that variations in the gene which encodes for the hepatic drug transporter, Organic Anion Transporter Polypeptide 1B1 (OATP1B1), *Solute Carrier Organic Anion Transporter Family Member 1B1* (*SLCO1B1)* (*rs4149056*, *rs2306283* and *rs4363657*), reduces statin uptake and metabolism and may be associated with statin intolerance. (Banach and others 2015; Bruckert and others 2005; Brunham and others 2012b; Cohen and others 2012; Kellick and others 2014a; Nissen and others 2016; Stroes and others 2015; Toth and others 2018; Zhang and others 2013)

Single nucleotide variations in the *SLCO1B1* gene leads to conformational changes in this transport mechanism, which may decrease hepatic uptake of statins (Hedenmalm and others 2010), and thereby decrease their metabolism, leading to potentially toxic concentrations of statins in their native forms in circulation. (Snpedia.com 2018) Single nucleotide variations may affect this transporter capability and influence how patients respond to statins. In fact, emerging research shows an association between statin intolerance and genetic variation. (Khine and others 2016; Link and others 2008a; Santos and others 2011; Snpedia.com 2018; Stewart 2013) Single nucleotide variations have been implicated in statin toxicity, in particular statin-intolerance, which is defined as statin-induced muscle related adverse events. (Khine and others 2016; Nagy and others 2015) Signs and symptoms associated with statin-intolerance include, muscle pain and weakness, muscle cramps, myositis and elevated CK levels. (Bruckert and others 2005; Cohen and others 2012; Nissen and others 2016; Stroes and others 2015; Zhang and others 2013)

Statin intolerance may vary in definition and severity. Currently there is no accepted definition of statin intolerance, but various definitions have been constructed by drug regulatory authorities, making the results from investigational studies difficult to compare. The definition used in this study, was that statin intolerance is a clinical

syndrome, which manifests as the inability to tolerate at least two statins, (one of which is at its lowest daily dose, 5 mg), due to symptoms and signs related to statin treatment, e.g., increase in laboratory markers and / or myopathy, which is agreed by the regulatory bodies (NAL, ILEP and CCWG). (Algharably and others 2017; Banach and others 2015; Jacobson and others 2014; Mancini and others 2016; Toth and others 2018)

Creatine kinase is expressed in high levels in the heart and skeletal muscle tissues. (Johnson and others 1989) Elevated plasma CK is therefore, one of the most commonly used biomarkers of statin-induced myopathy, indicative of myositis, myopathy and in severe cases, rhabdomyolysis. (Johnson and others 1989; Moghadam-Kia and others 2016) Levels of CK may be graded into three different classes: incipient myopathy, myopathy, and rhabdomyolysis. (Cham and others 2010; Wilke and others 2012)

When a statin tablet is administered orally, it dissolves in the stomach, is absorbed across the intestinal wall, travels to the liver via the portal circulation where it is metabolized and is either excreted via bile or transported to other tissues via the systemic circulation. (Tozer and Rowland 2006) During this process statins traverse various biological membranes, either by passive diffusion or by facilitated transport. Membrane transporters play a significant role here. (Kalliokoski and Niemi 2009) OATP1B1 is specifically known for its role in the hepatic uptake and clearance of statins, referred to as hepatobiliary excretion. Statins function as both a substrate and an inhibitor of this transporter. (König and others 2000; Niemi and others 2011)

Pharmacogenetic studies show that single nucleotide variations (SNVs), which are single base-pair mutations at specific sites in the human deoxyribonucleic acid (DNA) sequence, play an important role in the response and outcome of certain therapies. (Klug 2012; Kotlęga and others 2016) Of the numerous SNVs identified, SLCO1B1 rs4149056, rs2306283 and rs4363657 are the strongest contenders for genetic predisposition to statin-intolerance. (Khine and others 2016; Link and others 2008;

Santos and others 2011; Snpedia.com 2018; Stewart 2013) The most common and well characterized variants of *SCLO1B1* are *rs2306283* and *rs4149056*. These two variants appear to be in partial linkage disequilibrium, meaning these variants are more likely to be associated within a population than variants that are unlinked (Holloway and Prescott 2017) and most commonly occur in the four different haplotypes as presented in Table 1.

Table 1: Important haplotypes associated with rs2306238 and *rs4149056* (86)
Adapted from Oshiro *et al*. (2011)

Haplotype	Variant	Protein change
SLCO1B1*1a	Contains neither variant	-
SLCO1B1*1b	*rs2306283*	Asn130Asp
SLCO1B1*5	*rs4149056*	Val174Ala
SLCO1B1*15	Both	Val174Ala & Asn130Asp

Val (Valine), **Asn** (Asparagine), **Asp** (Aspartic acid), **Ala** (Alanine)

The SEARCH collaborative group (Link and others 2008) conducted a GWAS using archived DNA from a randomized control trial of more than 12 000 participants in order to identify SNVs in a group of 85 participants that could be linked to simvastatin intolerance. A significant association between rs4363657 and statin intolerance was identified. This non-coding SNV appeared to be in nearly complete linkage disequilibrium with two other well-known SNVs, located in the SLCO1B1 gene, namely rs4149056 and rs2306283. Of these SNVs, only rs4149056 located in the exon region appeared to be nonsynonymous, meaning it alters the encoded protein. Oshiro et al. (2011), however, found that both rs4149056 and rs2306283 are nonsynonymous SNVs. (Oshiro and others 2010) Furthermore, the risk associated with statin intolerance was found to be significantly higher in rs4149056 CC homozygotes than in T-allele carriers, (Link and others 2008) where T is the wild type allele. (Mombelli 2013; Stewart 2013)

The rs4149056 variant is associated with increased circulating concentrations of statins, notably simvastatin, including both the lactone prodrug and acid forms. (Oshiro and others 2010) For instance, a study revealed a 221% increase in the AUC and a 200% increase in the Cmax in patients with the homozygous (CC) genotype compared to those with the heterozygous wild type (TC) and homozygous wild type (TT) genotypes. (Pasanen and others 2006) Another single-dose study also showed significant increases in systemic drug exposure in patients with CC genotype. This data is presented in Table 2. (Wilke and others 2012)

Table 2: Increase in AUC of patients who present with CC genotype of *rs4149056* variant on SLCO1B1 gene

Data reported were obtained from a systematic review conducted by Wilke *et al*. (2012)(Wilke and others 2012)

Statin	**Increase in AUC**
Simvastatin acid	221%
Pitavastatin	162% – 191%
Atorvastatin	144%
Pravastatin	57% - 130%
Rosuvastatin	62% – 117%

The *rs2306283* variant on the *SLCO1B1* gene is also suspected of being associated with decreased activity of the *SLCO1B1* transporter, resulting in decreased clearance of statins from the circulation. (Lin and others 2011) However, the different haplotypes, i.e., particular combinations of alleles located on one of two homologous chromosomes at a nearby SNV, (Consortium 2003) associated with this SNV have yielded different results. (Santos and others 2012b) In the study on the effect of *SLCO1B1 rs2306283* *15 on the pharmacokinetic profiles of pravastatin and pitavastatin, a strong association between the altered transport of these statins and the specified haplotype was identified. (Deng and others 2008) However, two separate

studies to determine the association of this variant with statin intolerance indicated no significant association between *rs2306283* and atorvastatin and rosuvastatin induced myopathy, respectively. (Puccetti and others 2010; Santos and others 2012a) The data on the effect, as well as its combined effect with other statins is still inconclusive and limited. (Stewart 2013)

In South Africa, where health care is a disproportionate blend of first world (for a small minority) and developing (for the majority of the population) countries, healthcare is likely to change dramatically with the implementation of National Health Insurance. South Africa lags behind the rapid progress in personalized and precision medicine fields. Large-scale databases, technologically advanced methods to characterize patients and tools to analyze this data are required to improve diagnosis, therapeutic responses and ultimately health outcomes in this resource-limited setting.

Previous research has established the prevalence and associations of *SLCO1B1* (*rs4149056*, *rs2306283* and *rs4363657*) with statin intolerance in various populations, highlighting ethnic differences. (Dendramis 2011; Grigorova and others 2017; Li and others 2012; Luo and others 2015; Melo and others 2015; Méndez and others 2013; Musunuru and Kathiresan 2008; Nagy and others 2015; Nies and others 2013; Santos and others 2012) There is a paucity of research on these SNVs in South African populations. The aim of this study was therefore to determine the background prevalence of *SLCO1B1* SNVs in a randomly selected sample of the general population in Gauteng, South Africa, and to investigate if there are associations between *SLCO1B1* SNVs and hypercholesterolemia patients on statin therapy.

Materials and methods

Study design and participant selection

This was a cross sectional, explorative, experimental and retrospective study which included a quantitative questionnaire, of 181 control participants and 100 hypercholesterolemic patients. Participants included in the control group were

recruited from the general population and had never been diagnosed with hypercholesterolemia. Hypercholesterolemic patients were recruited from the clinical research unit of the University of Pretoria, and from public health institutions. Thus, the cohort was predominantly of a low-socio-economic population dependent on public health care.

Hypercholesterolemic patients were eligible for participation in this study if they were older than 18 years, provided written informed consent for study participation prior to the start of any study related procedures, were clinically diagnosed with hypercholesterolemia, and on a continuous and stable 12-week atorvastatin-/ simvastatin dose.

Healthy participants were eligible for participation in the control group if they provided written informed consent for study participation prior to the start of any study related procedures and if they were older than 18 years.

Questionnaire

A validated questionnaire based on the American College of Cardiology's (ACC) Statin Intolerance Application was used to evaluate statin intolerance in the 100 hypercholesterolemic patients who were receiving statin therapy. In 2013, the American College of Cardiology and the American Heart Association (ACC/AHA) developed a guideline for the treatment of hypercholesterolemia. (Stone and others 2014) In conjunction with this guideline, researchers developed an application through patient testing, and optimized by physicians and nurses, to provide a first line assessment for patients who present with statin intolerance. In this application, muscle symptoms are graded according to a scale of 0–10, where 0-2 is considered mild, 3-5 moderate and 6–10 severe. (Cardiology 2013; Pasternak and others 2002; Stone and others 2014) The questionnaire used in this study was adapted from this application. Statin intolerance risk was then calculated using the calculator provided by this online application. (Cardiology 2013)

Blood collection

Following informed consent, 5 ml blood samples were collected in a citrate tube by venipuncture from all study participants in order to evaluate their *SLCO1B1 rs4149056*, -*rs2306283* and -*rs4363657* SNV status. Whole blood (WB) samples were collected in 5 ml citrate tubes and centrifuged at 2 000 x *g* for 10 min. A total of 1 ml of serum was transferred to a collection tube and stored at -80ºC until DNA extraction.

DNA extraction

Genomic DNA was isolated from 200 µl of WB using the Quick-DNA™ Miniprep Plus Kit (Zymo Research).

Briefly, an aliquot of 800 µl cell lysis buffer was added to 200 µl WB in 1.5 ml microcentrifuge tubes. The tubes were vortexed for 10-15 seconds (s) and incubated at room temperature (RT) for 10 minutes (min). Following incubation, the sample was transferred into a Zymo-Spin™ IIC-XL column in a collection tube and centrifuged at 12 000 x *g* for 1 min. The collection tube with the flow through was discarded and the column was placed into a new collection tube. A total volume of 200 µl of DNA pre-wash buffer was added to the spin column and centrifuged at 12 000 x *g* for 1 min. The eluate collection tube was emptied, and 500 µl g-DNA wash buffer was added to the spin column and centrifuged at 12 000 x *g* for 1 min. The collection tube with the flow through was discarded. The spin columns were transferred into a clean 1.5 ml microcentrifuge tube, 50 µl DNA elution buffer was added directly on to the matrix and the sample was incubated for 15 min at RT. Following incubation, the tubes were centrifuged at 12 000 x *g* for 30 s to elute the DNA. The eluted DNA was stored at -20ºC and quantified using spectrophotometry (Nanodrop 2000c, Thermo-Fischer, South Africa), standardized to 10 ng/µl and used for polymerase chain reaction (PCR).

Polymerase Chain Reaction (Guo and others 2017; Nagy and others 2015)

Following optimization, *SLCO1B1 rs2306283*, *rs4149056* and *rs4363657* were amplified in a 25 µl. (Guo and others 2017; Nagy and others 2015) Primer concentrations were optimized to 400 nM. Primers for each SNV were as follows;

*SLCO1B1 rs2306283 (*F: 5'-CTGTGTTGTTAATGGGCGAA-3', R: 5'-GGGGAAGATAATGGTGCAAA-3'), *SLCO1B1 rs4149056 (*F: 5'-TTGTCAAAGTTTGCAAAGTG -3', R: 5'- GAAGCATATTACCCATGAGC -3') and *SLCO1B1 rs4363657(F: 5'-CAGTTTGCTAGTGTTTTGTTGAGG-3', R: 5'-ACCATCCAAGACGAACAAAGAG -3').* Restriction enzymes were, *TaqI, Hin6I* and *KpnI,* respectively.

The PCR conditions for *SLCO1B1 rs2306283* were pre-denatured for 2 min at 95°C, followed by a three-step amplification, denaturation at 95°C for 45 s, annealing at 55.5°C for 45 s and extension at 72°C for 45 s. This was followed by a final extension at 72°C for 300 s and cooling at 37°C for 300 s. For *SLCO1B1 rs4149056,* were pre-denatured for 2 min at 95°C, followed by a three-step amplification, denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 45 s. This was followed by a final extension at 72°C for 300 s and cooling at 37°C for 300 s. Pre-denaturation for *SLCO1B1 rs4363657* was for 2 min at 96°C, followed by a three-step amplification, denaturation at 95°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 45 s. This was followed by a final extension at 72°C for 300 s and cooling at 37°C for 300 s. (Guo and others 2017; Nagy and others 2015)

Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

The restriction of the SNVs described in Table 2 were conducted in a 25 µl reaction (3 µl PCR product, 5 µl 1X New England (NE) buffer and 1 µl RE (1 000U)) (New England BioLabs) using the restriction enzymes specified in Table 2. The restriction fragments were then analyzed using gel electrophoresis on a 3% agarose gel for the *SLCO1B1* SNVs for 30 min and then visualized with a ultra violet (UV) transilluminator (Gel Doc EZ Imager (BioRad, version 5.21))

Quantitative measurement of Creatine Kinase in plasma

Creatine kinase levels were estimated using the CKM Human SimpleStep ELISA® Kit:

Briefly, plasma was diluted with Sample Diluent in a 1:50 ratio. Standards were reconstituted and prepared in sample diluent. A total of 50 µl of each sample or

standard with known concentration (0 pg/mL, 62.5 pg/ml, 125 pg/ml, 250 pg/ml, 500 pg/ml, 1000 pg/ml, 2000 pg/ml, 4000 pg/ml) was added in duplicate to the appropriate wells in a 96 well plate, after which 50 µl of the antibody cocktail was added to each well. The plate was sealed and incubated for 1 hour at RT on a plate shaker set to 400 rpm. After incubation, each well was washed with 3 x 350 µl 1 x Wash Buffer PT by aspirating from each well and then dispensing 350 µl 1 x Wash Buffer PT into each well. A total of 100 µl of TMB substrate was added to each well (5 min, RT, 400 rpm). After incubation, 100 µl of Stop Solution was added to each well and the plate was placed back onto the plate shaker for 1 min to ensure thorough mixing. The optical density was measured using a spectrophotometer (Biotek Synergy HT, Software GEN 5.1) at an absorbance 450 nm. A standard curve was constructed, and concentrations of the unknown samples were calculated using the equation derived from the standard curve (y=mx+c).

Statistical analysis

A Mann Whitney U-test was conducted to determine differences between demographic parameters. SNVs in a hypercholesterolaemic population were reported using appropriate descriptive and inferential statistics and the Hardy-Weinberg equilibrium (HWE) test. Fisher's exact test was used to determine the odds ratio (OR) and relative risk (RR) for each SNV. Analysis for CK levels included column statistics using the recommended D'Agostino and Pearson test to determine if the data followed a normal distribution. Comparisons between groups were achieved using the non-parametric tests (Mann Whitney U-test and one-way ANOVA (Kruskal-Wallis test with Dunn's post-test for multiple comparisons between groups). The level of significance was set at $p<0.05$. The analysis was carried out on GraphPad Prism v6 (San Diego, California).

Results

Cohort demographics

The cohort included 281 participants between the ages of 19 and 75. Of these, 100 were included into the hypercholesterolaemic group and 181 in the control group. The male to female ratios were similar between the hypercholesterolemic and control

groups. The full cohort demographics are shown in Table 3. Most (70%) of the hypercholesterolemic patients were treated with the first line statin, simvastatin.

Table 3: Participant demographics

	Hypercholesterolemic group (*n*=100)	**Control group (*n*=180)**
Age median (range) in years	51 (19 – 75)	49 (20 – 75)
Sex		
Male (%)	49 (49%)	89 (49.2%)
Female (%)	51 (51%)	92 (50.8%)
Race		
Black (*n*)	86	142
White (*n*)	14	39
Comorbidities		
Rheumatoid arthritis (*n*)	2	0
Psoriatic arthritis (*n*)	4	0
Osteoarthritis (*n*)	9	0
Type 2 Diabetes Mellitus (*n*)	12	0
Hypothyroidism (*n*)	2	0
Low body mass index (*n*)	2	0
Treatment group		
Atorvastatin (*n*)	29	0
Simvastatin (*n*)	71	0

Statin intolerance risk score

All participants in the hypercholesterolemic group (n=100) completed a detailed questionnaire adapted from the American College of Cardiology's (ACC) Statin Intolerance Application. The questionnaire collected data on muscle symptoms, their severity, frequency, patient characteristics and medical history that may increase a patient's predisposition for statin intolerance. Table 4 illustrates the distribution of patients on different statin treatments as high, moderate, or low-risk. Of the 100 patients in the hypercholesterolemic group, four had risk factors that may worsen or contribute to statin intolerance, i.e. low body mass index (BMI) and hypothyroidism. All of these patients demonstrated moderate risk for statin intolerance with two of the four carrying at least one of the assessed variants.

Table 4: Statin intolerance risk score

Statin intolerance risk score	**Treatment groups**				
	Atorvastatin 10 mg	Atorvastatin 20 mg	Simvastatin 10 mg	Simvastatin 20 mg	Simvastatin 40 mg
High risk (*n*)	-	2 (10%)	7 (26%)	4 (11%)	2 (29%)
Moderate risk (*n*)	4 (40%)	12 (60%)	13 (48%)	16 (44%)	4 (57%)
Low risk (*n*)	6 (60%)	6 (30%)	7 (26%)	16 (44%)	1 (14%)
Total	10 (10%)	20 (20%)	27 (27%)	36 (36%)	7 (7%)

Single nucleotide variations

The statin-treated hypercholesterolemic (test) group genotype distribution conformed with the Hardy-Weinberg equilibrium (HWE) (p>0.05). However, the *SCLO1B1 rs4149056* and *rs2306283* genotype distribution in the control group did not conform with the HWE (p<0.05), in the total cohort and for blacks only. The prevalence of the *rs4149056* variant was 16% for the control group and 20% for the test group but was not statistically significant. (Odds ratio (OR)=1.324; 95% confidence interval

(CI)=0.8430 to 2.078; *p*=0.2405). Conversely, the *rs2306283* variant was significantly more prevalent in the control group compared to the test group (31.5% compared to 10.5%, OR=0,2552 95% CI=0.1542 to 0.4223, *p*< 0.0001. The prevalence of the *rs4363657* variant was similar in both the test and control group. (OR=1.345, 95% CI=0.8492 to 2.129, *p*=0.2380). Table 5 illustrates the frequency and distribution of the *SLCO1B1 rs4149056, rs2306283* and *rs4363657* SNVs.

Table 5: Genotype and Allele frequencies						
Population	**n**	**Homozygous wild type n *(%)***	**Heterozygous n *(%)***	**Homozygous variant n *(%)***	**Presence of variant n *(%)***	**P value[1]**
rs4149056	**281**	**TT**	**TC**	**CC**	**C allele**	
Control	181	138 *(76.7)*	30 *(16.6)*	13 *(7.3)*	56 *(15.5)*	*p*= 0.2405
Test	100	62 *(62)*	37 *(37)*	1 *(1)*	39 *(19.5)*	
rs2306283	**281**	**AA**	**AG**	**GG**	**G allele**	
Control	181	95 *(52.5)*	58 *(32)*	28 *(15.5)*	114 (*31.5)*	*p*< 0.0001
Test	100	79 *(79)*	21 *(21)*	0 *(0)*	21 *(10.5)*	
rs4363657	**281**	**TT**	**TC**	**CC**	**C allele**	
Control	181	140 *(77.3)*	40 *(22.1)*	1 *(0.6)*	42 *(11)*	*p*= 0.2380
Test	100	60 *(60)*	38 *(38)*	2 *(2)*	42 *(21)*	

[1] *P*-values were derived from fisher's exact test

To eliminate the effect race admixture might have on the genetic analysis present in Table 5, analysis was done to evaluate the prevalence of each SNV in the black participants which makes up the majority of the sample as shown in Table 5. The prevalence of the *rs4149056* variant was 15% for the control group and 22% for the test group but was not statistically significant. (Odds ratio (OR)= 0.6685; 95% confidence interval (CI)= 0.4151 to 1.077; *p*=0.1055). The *rs2306283* variant was

significantly more prevalent in the control group compared to the test group (33% compared to 9%, OR= 0.1948 95% CI= 0.1090 to 0.3481, *p*< 0.0001). The prevalence of the *rs4363657* variant was significantly higher in the control group compared to the test group. (OR= 2.086, 95% CI= 1.261 to 3.450, *p*=0.0051). Table 5 illustrates the frequency and distribution of the *SLCO1B1 rs4149056, rs2306283* and *rs4363657* SNVs in the black participant group.

Table 6: Genotype and Allele frequencies (Blacks only)

Population	**n**	**Homozygous wild type n (%)**	**Heterozygous n (%)**	**Homozygous variant n (%)**	**Presence of variant n (%)**	**P value[1]**
rs4149056	**241**	**TT**	**TC**	**CC**	**C allele**	
Control	155	118 (76)	26 (17)	11 (7)	48 (15)	*p*= 0.1055
Test	86	50 (58)	35 (41)	1 (1)	37 (22)	
rs2306283	**241**	**AA**	**AG**	**GG**	**G allele**	
Control	155	78 (50)	52 (34)	25 (16)	102 (33)	*p*< 0.0001
Test	86	71 (83)	15 (17)	0 (0)	15 (9)	
rs4363657	**241**	**TT**	**TC**	**CC**	**C allele**	
Control	155	119 (77)	36 (21)	0 (0)	274 (88)	*p*= 0.0051
Test	86	51 (59)	33 (38)	2 (2)	135 (78)	

[1] P-values were derived from fisher's exact test

Table 4 shows 2 (7%) of the atorvastatin-treated patients had a high risk of developing statin intolerance, 15 *(52%)* had a moderate risk of statin intolerance and 12 *(40%)* a low-risk of statin intolerance. Of the simvastatin-treated patients,13 *(19%)* presented with high risk to statin intolerance, 33 *(47%)* with moderate risk to statin intolerance and 24 *(34%)* with low risk to statin intolerance. The wild type genotype was more

prevalent than the variant for all SNVs. The variant allele for *rs2306283* was more prevalent in the control group than in the test group at 31.5% and 10.5% respectively.

The effect of the presence the *SLCO1B1* single nucleotide variations on statin intolerance

Following the determination of the frequency and prevalence of each variant and the statin intolerance severity risk score of each included patient, an analysis was performed to ascertain if there was an association between the presence of either of the single nucleotide variants and statin intolerance risk in order to assess if a genotype had an effect on muscle fatigue and muscle weakness. The genotype frequency for each SNV was compared within two groups, namely low risk statin intolerance and moderate to high risk statin intolerance. This analysis showed no significant association for any of the three SNVs and the presentation as either low risk or moderate to high risk statin intolerant, *rs4149056* (OR=0.7857, 95% CI=0.2115 to 2.919, relative risk (RR)= 0.8800, 95% CI= 0.4433 to 1.747, *p*=0.7496) and *rs2306283* (OR=0.4911, 95% CI=0.1234 to 1.954, RR=0.9659, 95% CI 0.4888 to 1.909, *p*=0.4877) and *rs4363657* (OR= 0.9375, 95% CI= 0.2634 to 3.3337, RR=0.6984, 95% CI=0.3609 to 1.352, *p*=1.0000).

There was no difference in the frequency of either variant between moderate to high risk to statin intolerance and patients with a low risk of statin intolerance. For *rs4149056*, low-risk wild type frequency was 28.3% compared to 35.9% in the moderate to high risk and the variant (C-allele) occurred 17.9% times in both groups. For *rs2306283*, the variant allele was present in 17.9% of the low-risk participants and in 12.8% or moderate to high-risk participants, while the wild type of genotype was present in 28.3% and 41% of the low risk and moderate to high-risk patients, respectively. For *rs4363657*, the wild type genotype was seen in 25.6% of the low-risk participants and 30.8% of the moderate to high-risk participants while the variant allele was only present in 20.5% of the low risk and in 23.1% of the moderate to high-risk participants.

Quantification of Creatine kinase (CKM)

The average CKM level in the atorvastatin group was 16.52 ng/ml compared to 26.74 ng/ml in the simvastatin group (Figure 1). The CKM levels of patients on simvastatin were significantly higher compared to those on atorvastatin (p=0.0418).

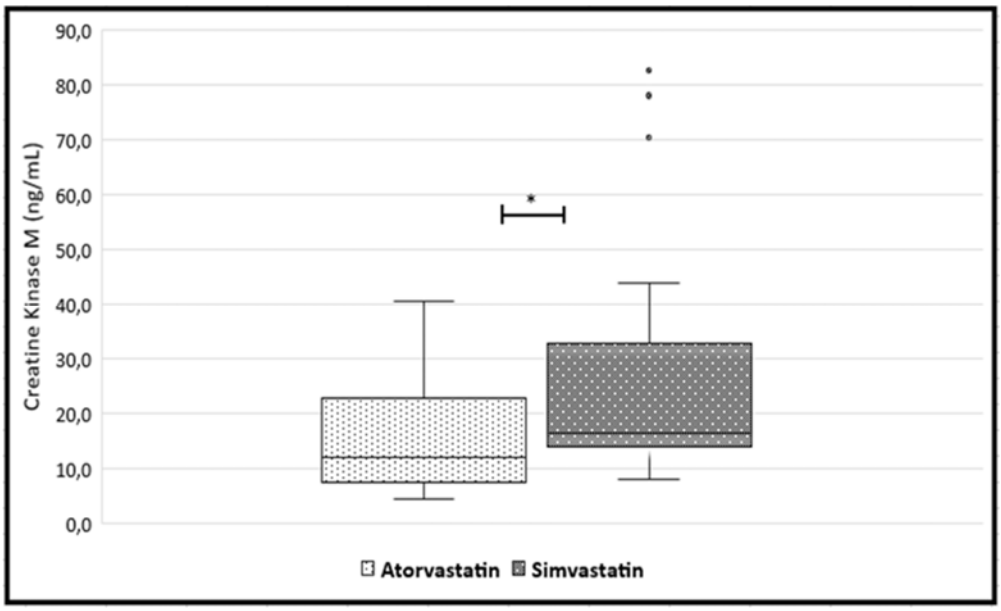

Figure 1: Serum CKM levels between patients on simvastatin and atorvastatin therapy *p= 0.0418, Mann-whitney U test.

Determining if there are associations between symptoms and signs of statin intolerance and individual SNVs.

The estimated CKM levels of each patient within each haplotype (group 1: moderate/high risk with 3 SNVs present; group 2: moderate/high risk with SNV *rs4149056* and SNV *rs4363657*; group 3: 3 moderate/high risk with SNV *rs2306283* and SNV *rs4363657*; group 4: moderate/high risk with SNV *rs4149056* and SNV *rs2306283*; group 5: low risk with 1 SNV only and group 6: low-risk wild type) were compared. The range in CKM levels for each haplotype was; Group 1: 4.57 ng/ml – 15.65 ng/ml, Group 2: 6.15 ng/ml – 37.48 ng/ml, Group 3: 12.32 ng/ml – 32.73 ng/ml, Group 4: 15.57 ng/ml – 34.15 ng/ml, Group 5: 10.65 ng/ml – 44.07 ng/ml, Group 6: 7.48 ng/ml – 40.65 ng/ml and no significant difference between CKM levels was found (p=0.2048, one-way ANOVA with Kruskal-Wallis multiple comparisons and Dunn's post-test).

The CK levels of patients with wildtype genotypes for all SNVs and those that presented with low-risk statin intolerance when compared with patients who presented with moderate to high-risk statin intolerance and the presence of at least 1 SNV were not statistically different (p=0.9885) (Figure 2).

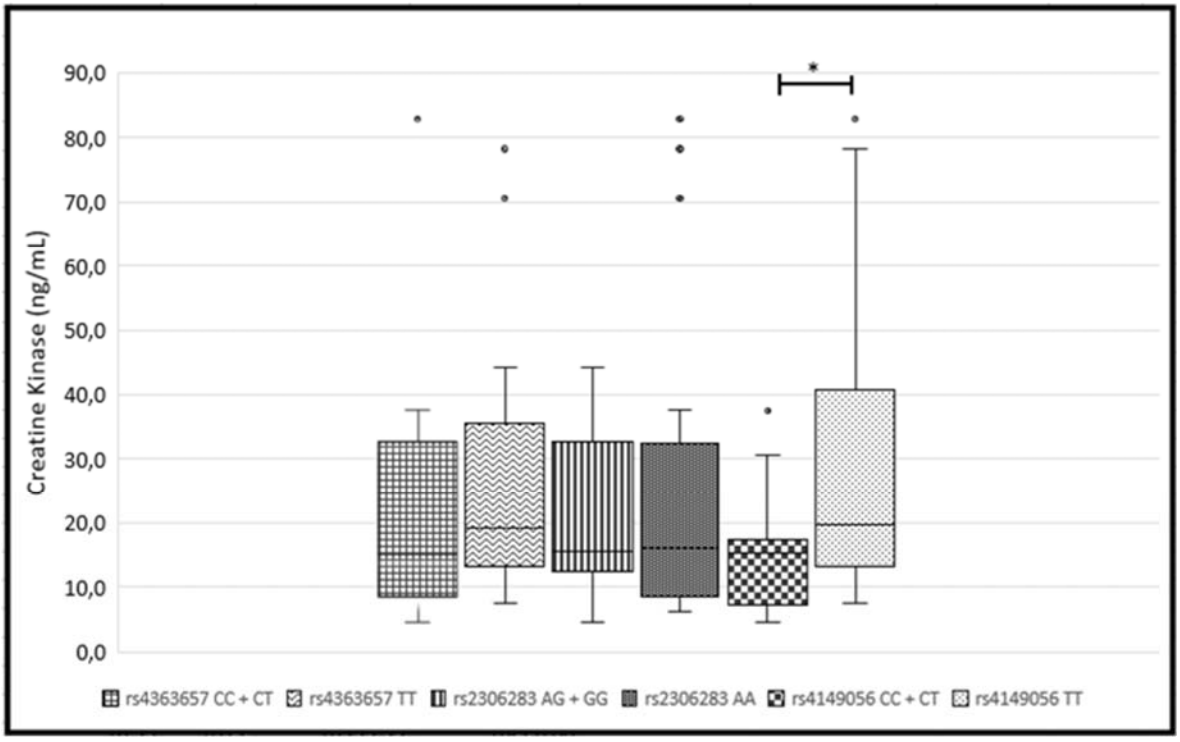

Figure 2: Levels of CKM in wild-type and in the presence of the variant genotype, for rs4363657, rs2306283 and rs4149056. *Where p<0.05, Mann-whitney U-test .

Discussion

Single nucleotide variations have been identified in *SLCO1B1 rs4149056*, *rs2306283* and *rs4363657* and are believed to influence statin intolerance. (Nagy and others 2015) A global analysis of the genetic variation in *SLCO1B1* stated that the *rs4149056* has an allele frequency of ~15 – 20% in Caucasian populations and a ~1 – 2% in Black populations. The *rs2306283* variant has a frequency of ~40% in Caucasian populations with little to no data on Black populations. The *rs4363657* variant illustrated a high allelic frequency in Caucasian, East African and African American populations of ~30%, (Dendramis 2011; Grigorova and others 2017; Hubáček and others 2015; Lee and others 2005; Li and others 2012; Liutkeviciene and others 2018;

Luo and others 2015; Melo and others 2015; Méndez and others 2013; Musunuru and Kathiresan 2008; Nagy and others 2015; Nies and others 2013; Pasanen and others 2006a; Santos and others 2012; Tirona and others 2001) There is a paucity of research on the prevalence of these SNVs in the diverse South African population.

This research study is the first to report the presence of *SLCO1B1* SNVs in a South African population. Of note is that only Black and Caucasian patients were included in the study in an attempt to provide a close representation of the population ethnic ratio in the region. The prevalence of the variant allele for the total cohort was 15.1% for *rs4363657*, 24% for *rs2306283* and 17.4% for *rs4149056*. The prevalence of each SNV varied between ethnicities and no difference was found for *rs4363657* and *rs2306283*. The frequency of the *rs4149056* variant was significantly higher amongst the Black participants with hypercholesterolemia (53.3%) compared to Caucasians (15.3%), p=0.0206, OR: 3.467. Interestingly, the prevalence of both the rs2306283 and rs4363657 variant allele were significantly more prevalent in the control group compared to the test group of the black population, p <0.0001 and p= 0.0051, respectively. (Table 5)

Other studies have also shown diverse results in Asian, European, North and South American and East African populations. Agnes Nagy (2015) explored the differences in frequencies of *SLCO1B1* variants between Roma (n= 470) and Hungarian (n=442) populations and found the *rs2306283* variant to be the most prevalent in both ethic groups (presence of G allele/variant 54.5% and 36.2%). The *rs4149056* variant was evident in 17.2% of the Roma participants and 18.9% of the Hungarian participants, while the *rs4363657* variant appeared to be the least frequent with the variant in only 19.8% and 19.5% of the Roma and Hungarian participants, respectively. (Nagy and others 2015) Mwinyi *et al*. (2008) (Mwinyi and others 2008) found that the prevalence of the *SLCO1B1* gene variations were low in a selected African cohort from Uganda compared to other populations. Eighteen *SLCO1B1* variants were explored in this study. However, only 6 were present in the African cohort. Prevalence of the *rs4149056* variant was only 3.9%, but the prevalence of the *rs2306283* variant was significantly higher at 77.8% (95% CI= 71.9 -83.0). (Mwinyi and others 2008) In this

current study, prevalence of the *rs4149056* variant (16.9%) was similar to the prevalence of the *rs2306283* variant (24%).

The SNVs most commonly and widely associated with statin intolerance are *rs2306283* and *rs4149056*. These SNVs are in linkage disequilibrium and are most commonly identified together in the same population. (Holloway and Prescott 2017) In this study, the *rs4149056* variant was more prevalent in the hypercholesterolaemic (test) group compared to the control group. The *rs2306283* variant was most commonly identified in the control group. In this study, the presence of both *rs4149056* and *rs4363657* were more common amongst subjects in the control than test groups. This result is substantiated by the GWAS conducted by the 'SEARCH collaborative' in 2008 which concluded that *rs4149056* and *rs4363657* are in complete linkage disequilibrium. (Link and others 2008b)

The majority (64%) of the hypercholesterolemic patients in this study reported muscle-related adverse effects. Most (49%) reported their muscle pain as mild to moderate. Moderate severity indicates that muscle related symptoms only slightly reduce everyday activities such as experiencing difficulty in working, sleeping, performing household chores and climbing stairs. Reducing the statin dose may be a feasible treatment option while monitoring these patients' CK levels. (Kellick and others 2014a) A total of 17% reported severe pain. Other studies have reported an overall prevalence of ~30% of severe myalgia, myopathy and in some cases rhabdomyolysis. In these reports, the therapy included various statins, with myopathy observed in subjects on simvastatin and atorvastatin. (Ballantyne and others 2003; Group 2002; Jones and others 2003; Jones and others 1998; Newman and others 2003; Scandinavian Simvastatin Survival Study 1994)

The risk of developing statin intolerance was found to be low (in 36%), moderate (in 49%), or high (in 15%) in the statin-treated hypercholesterolemic patients and was based on what was reported in the questionnaires. The prevalence in the latter two

categories (64%) correlated well with the actual development of muscle-related adverse effects (65%).

The questionnaire also included analysis of possible drug-drug interactions from concomitant medications that might lead to adverse effects observed with statin treatment. However, none of the participants included in the hypercholesterolemia group were prescribed contraindicated medications. Thus, no correlation could be drawn between the adverse events associated with statin intolerance and drug-drug interactions.

A total of 10% of patients had elevated plasma CK, compared with 64% of patients who reported muscle symptoms. Ballantyne *et al*. (2003) (43) described various symptoms reported by patients on simvastatin and atorvastatin. These ranged from low grade muscle cramps and body aches to severe muscle weakness. However, these symptoms were not accompanied by a significant plasma CK elevation. (43) Although statin intolerance is not always accompanied by extreme elevation in the CK level (> 10 to 50 x ULN), a slight increase can result from myopathy and muscle breakdown. (Toth and others 2018). This highlights a consistent lack of correlation between CK levels and signs and symptoms of statin intolerance.

The highest CKM levels recorded in this study were 70.57 ng/ml, 78.23 ng/ml, and 82.73 ng/ml. Of these, the patient who presented with the highest CKM level (82.73 ng/ml), carried the variant for both the *rs4149056* and *rs4363657* SNVs, but reported little to no muscle related symptoms. Furthermore, CK analyses indicated a slight (27.7%), elevation in the median CK level of the low risk hypercholesterolemic participants who presented with only 1 of the 3 SNVs. These differences, however, were not statistically significant.

Another objective was to determine and compare possible associations of SNVs and elevated CKM levels between simvastatin and atorvastatin subgroups. Although all

statins are substrates of the OATP transporter, the effect the *SLCO1B1* SNVs has on the pharmacokinetics differs between statins. This study compared the effect of simvastatin and atorvastatin on CKM levels and statin intolerance in order to determine which statin is safer in patients who carry any or all of the three SNVs.

The majority of participants were on 20 mg atorvastatin (20%), 10 mg simvastatin (27%) or 20 mg simvastatin (36%). While this study did not specifically assess the pharmacokinetic influence of individual SNVs, CKM analysis did show that the CKM level of hypercholesterolemic patients on simvastatin was significantly higher compared to atorvastatin. Simvastatin yielded a mean CKM level of 26 744 pg/ml, almost double the mean 16 517 pg/ml CK level for atorvastatin. This finding reiterates that atorvastatin might be a safer and more tolerable option for hypercholesterolemic patients.

This is corroborated by other studies. (Carr and others 2013; Link and others 2008a) Carr *et al*. (2013) included 76 statin-induced myopathy cases reported between June and November of 2011, of which 59 were receiving simvastatin, 11 atorvastatin and 6 other statins. Their findings indicated that having just one allele of the *rs4149056* variant resulted in severe myopathy and an elevation in the CK level of at least four times the upper normal limit (UNL) in simvastatin treated patients. Although atorvastatin was their second most implicated drug, there was no significant association between the *SLCO1B1* variant and significant elevations of CK or severe myopathy. (Carr and others 2013) These results (Link and others 2008a) were comparable with those found in the GWAS conducted by Link *et al*. (2008) and the study by Brunham *et al*. (2011) which aimed to investigate the statin-specificity of the association between *SLCO1B1* variations and severe statin-induced myopathy. (Brunham and others 2012a; Brunham and others 2012b; Link and others 2008a) In both these studies it was found that although the *SLCO1B1* variations might not specifically be associated with statin-induced myopathy, there was a significant association (Brunham at al.(2011)(Brunham and others 2012a) OR= 3.2, 95% CI 0.83–11.96, χ^2 p= 0.042, Fisher's exact p= 0.064 and Link *et al*. (2008)(Link and others 2008a) OR= 4.3 (95% CI, 2.5 to 7.2)) between the patients receiving simvastatin and

myopathy. (Brunham and others 2012a; Link and others 2008a) This finding parallels the findings from the current study. A study by Pasanen *et al*. (2006) (Pasanen and others 2006b) that aimed to assess the effect that transporter variations have on statins, reported that the *SLCO1B1* variations had the greatest effect on simvastatin uptake. The researchers specifically investigated *rs4149056* and demonstrated that the presence of the variation markedly affected the pharmacokinetic profile of the drug. The Cmax was 221% higher in participants with the variation compared to those without. (Pasanen and others 2006b) A GWAS study conducted in 2008 identified SNVs in a group of 85 participants that could have an association with statin intolerance. A significant association was drawn between *rs4363657* and *rs4149056* and statin induced myopathy. After genotyping both SNVs, the risk associated with simvastatin intolerance was significantly higher in patients who presented with the homozygote (CC) genotype compared to the T allele carriers. (Link and others 2008a; Mombelli 2013; Stewart 2013)

Previous research has established an association between *SLCO1B1* (s4149056, *rs2306283* and *rs4363657*) and statin intolerance in various populations, highlighting ethnic differences in the prevalence of these variations. (Dendramis 2011; Grigorova and others 2017; Li and others 2012; Luo and others 2015; Melo and others 2015; Méndez and others 2013; Musunuru and Kathiresan 2008; Nagy and others 2015; Nies and others 2013; Santos and others 2012) For instance, Lin *et al*. (2011) (Lin and others 2011) identified the *rs2306283* and *rs4149056* variants on the *SLCO1B1* gene as two of the *SLCO1B1* variants most commonly associated with decreased activity of the OATP1B1 transporter, resulting in a decreased clearance of statins from the circulatory system and eventual statin intolerance. (Lin and others 2011) Despite evidence in other populations, statistical analyses indicated no significant association between the signs and symptoms associated with statin intolerance and the presence of an SNV in this cohort.

Comorbidities in 31% of the study population included hypothyroidism, T2DM, rheumatoid arthritis, osteoarthritis, psoriatic arthritis, and small body frame (low BMI). These are risk factors that could initiate or worsen symptoms associated with statin

induced myopathy, potentially confounding the data. (Bar and others 2007; Kajinami and others 2005) Despite this, only a minority (13%) of patients with comorbidities were considered to have a high risk of developing statin intolerance. However, this may have been due to the statin dose (mostly simvastatin 10 – 40 mg), which is currently considered the most significant risk factor for developing statin intolerance. (Jones and others 1998)

Conclusion

In this study, no association between the presence of SNV, signs and symptoms of statin intolerance and elevated CK levels was found. However, this study showed conclusively that simvastatin treated patients had a higher risk of statin induced myopathy compared to the atorvastatin treated patients as confirmed in many other studies. (Brunham and others 2012a; Brunham and others 2012b; Carr and others 2013; Link and others 2008a)

An estimated 1 in 75 South Africans is hypercholesterolemic. (Health24 2020) The results in this study, provide a better understanding and are the first to explore the prevalence of these SNVs and their association with hypercholesterolaemia in a South African population. These findings will facilitate a more personalized approach to statin therapy, especially relevant within the diverse South African population. This prompts a need to investigate a larger population, especially to determine if the presence of SNVs affects CK levels, and to determine whether or not there is a differential effect in patients who present with the homozygous variant compared to heterozygous or homozygous wild types.

Limitations

Participants on different statin doses were included in the study which could also lead to statin-related side effects occurring less frequently in participants on lower statin doses.

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