Genomic Diversity and Functional Analysis of the Solute Carrier Genes within Indigenous African and Cape Admixed Populations

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A thesis submitted in partial fulfilment of the requirements for the degree of *Philosophiae Doctor* in the Department of Biotechnology, University of the Western Cape.

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LIST OF PUBLICATIONS

**Denotes first author and/or equal authorship.*

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2. ***Hoosain N, *Pearce B, Jacobs C, Benjeddou M**. (2016) Mapping SLCO1B1 Genetic Variation for Global Precision Medicine in Understudied Regions in Africa: A Focus on Zulu and Cape Admixed Populations. OMICS. 20(9):546-54. doi: 10.1089/omi.2016.0115.
3. ***Du Plessis, M., *Pearce, B., Jacobs, C., Hoosain N. and Benjeddou, M**. (2015) Genetic polymorphisms of the organic cation transporter 1 gene (SLC22A1) within the Cape Admixed population of South Africa. *Molecular Biology Reports* **42**: 665-672

ABSTRACT

Solute carrier transporters belonging to the major facilitator family of membrane transporter are increasingly being recognized as a possible mechanism to explain inter-individual variation in drug efficacy and response. Genetic factors are estimated to be responsible for approximately 15-30% of inter-individual variation in drug disposition and response. The aims of this study were to determine the minor allele frequencies of 78 previously identified single nucleotide polymorphisms in the pharmacogenomically relevant *SLC22A1-3* and *SLCO1B1* genes in the Admixed population of South Africa. Thereafter, to determine whether allele and genotype frequencies for these SNP were different from that reported for other African, Caucasian, and Asian populations. The inferred haplotypes from the genetic information possessed the potential to subsequently be used in future to design and interpret results of pharmacogenomic association studies involving these genes and their substrate drugs. Furthermore, to determine whether the Cape Admixed population harbour novel SNPs in the proximal promoter regions of *SLC22A1-3* and SLCO1B1-3 genes, that encodes hOCT1-3 and hOATP1 and hOATP3, respectively. SNaPshot™ multiplex single base mini-sequencing systems were developed and optimized for each of *SLC22A1*, *SLC22A2*, *SLC22A3*, and *SLCO1B1* genes covering the previously identified 78 SNPs. These systems were then used to genotype the alleles of 130 healthy Cape Admixed subjects residing in Cape Town, South Africa. In addition, the proximal promoter regions of the *SLC22A1-3* and *SLCO1B1-3* genes of 96 of the participants were screened for novel SNPs by direct sequencing. The Cape Admixed subjects investigated displayed a lack of variation and were monomorphic for 78% of

the SNPs screened. None of the *SLC22A3* SNPs investigated was observed in this study. Sequencing of the proximal promoter regions of the *SLC22* and *SLCO* genes did not reveal any novel SNPs in the 96 Cape Admixed subjects that were screened. This study highlights the fact that African populations do not have the same allele frequencies for SNPs in pharmacogenomically relevant genes. Furthermore, the Cape Admixed and other African populations do not share all reduced-function variants of the *SLC22A1-3* and *SLCO1B1-3* genes with Caucasian and Asian populations. In addition, previously identified novel regulatory variants in *SLC22A2* did not exhibit a significant effect on the ability of the promoter to drive transcription. However, it must be noted that these results were observed at 95% confidence interval, and that a 99% confidence interval the significance may increase theoretically. Additionally, it should be noted that more intensive studies are required to determine the potential effect these novel variants may well cause. This study lays the foundation for the design and interpretation of future pharmacogenomic association studies between the variant alleles of the *SLC22A* and *SLCO* genes in the Cape Admixed population, as well as optimizations for future expression, and more importantly, drug transport assays with respect to drug disposition and efficacy.

DECLARATION

I declare that '*Genomic Diversity and Functional Analysis of the Solute Carrier Genes within Indigenous African and Cape Admixed Populations*' is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

BRENDON CLIVE PEARCE NOVEMBER 2016

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LIST OF ABBREVIATIONS

AA	Amino Acid
ADR	Adverse Drug Reactions
AMPK	Adenosine Monophosphate-activated Protein kinase
BLAST	Basic Alignment Tool
CAM	Calmodulin
CCC	Cholangiocellular Carcinoma
CML	Chronic Myeloid Leukemia
dbSNP	Database of Single Nucleotide Polymorphisms
DDI	Drug-Drug Interaction
DNA	Deoxyribonucleic Acid
EMT	Extraneuronal Monoamine Transporter
GWAS	Genome-Wide Association Studies
HCC	Hepatocellular Carcinoma
HNF	Hepatocyte Nuclear Factor
MATE	Multidrug and Toxin Extrusion
MAF	Minor Allele Frequency
MPP^+	1-Methyl-4-Phenylpyridinium
mRNA	Messenger Ribonucleic Acid
NCBI	National Centre for Biotechnology Institute
OCT	Organic Cation Transporter

PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator Agonist Receptor
SHP	Small Hetero-dimer Partner
SLC	Solute Carrier Transporter
SNP	Single Nucleotide Polymorphism
TEA	Tetraethylammonium
TMH	Trans-Membrane Helix
TKI	Tyrosine Kinase Inhibitor
USF	Upstream Stimulating Factor

LIST OF FIGURES

		Page
Figure 1.1	Multiple protein sequence alignment of OCTs from four animal species showing a high degree of evolutionary conservation.	**14**
Figure 1.2	Predicted membrane topology of OCTs as represented by human OCT1.	**15**
Figure 1.3	Chemical structures of 4 platinum agents which are substrates of hOCTs and hMATEs.	**16**
Figure 1.4	Chemical structures of selected bio-amine substrates of hOCTs.	**17**
Figure 1.5	Schematic representation of transcriptional and post-translational regulation of hOCT1 (A) and hOCT2 (B).	**18**
Figure 1.6	Multiple protein sequence alignment of MATE protein sequences from four different animal species.	**20**
Figure 1.7	A schematic representation of the gene organization and the primary protein structure of human (A) *SLC47A1* (hMATE1) and (B) *SLC47A2* (hMATE2-K). The diagram depicts exons (numbered 1-17) and introns of both genes together with the arrangement of transmembrane helices (TMHs) (numbered 1-13) of encoded proteins.	**22**
Figure 1.8	Pharmacokinetics pathway of metformin.	**34**
Figure 2.4.1	Allele frequencies of selected *SLC22A1* SNPs in the CA population compared to other African and world populations.	**70**
Figure 2.4.2	Typical electropherogram of *SLC22A2* multiplex 1 mini-sequencing fragments. Black represents C, green A, blue G, and red T.	**72**
Figure 2.4.3	Allele frequencies of selected *SLC22A2* SNPs in the CA population compared to other African and world populations.	**76**
Figure 3.4.1	Allele frequencies of selected *SLCO1B1* SNPs in the CA population compared to other African and world populations.	**104**

Figure 4.4.1 Minor allele frequencies of selected promoter SNPs in the CA population compared to other African and world populations. **120**

Figure 5.2.1 Electropherograms of novel SNPs observed in the proximal promoter region of the *SLC22A2* gene. MBPG_OCT2001 (wild-type -156 G/G and variant -156 G/A) a novel SNP detected in the proximal promoter region. **130**

Figure 5.4.1 Confirmation of GFP expression by fluorescent microscopy. A) HEK293 cells expressing GFP with cloned -95 haplotype. B) LNCaP cell expressing GFP with -95 haplotype. **133**

Figure 5.5.1 Relative fluorescence detected by flow cytometry. A) Untransfected HEK293 cells. B) HEK 293 cells transfected with the positive control, expressing GFP. Where M11 indicates cell debris and M13 indicates fluorescence. **136**

Figure 5.5.2 Fluorescent measurement of transfected HEK293 cells, where M11 indicates cell debris and untransfected cells, and M13 is indicative of fluorescence. A) positive control expressing GFP. B) -95 Haplotype, transfected cells expressing GFP. C) -156 Haplotype, transfected cells expressing GFP. **137**

Figure 5.5.3 Fluorescent measurement of transfected LNCaP cells, where M11 indicates cell debris and M13 is indicative of fluorescence. A) -95 Haplotype, transfected cells expressing GFP. B) -156 Haplotype, transfected cells expressing GFP. **137**

LIST OF TABLES

		Page
Table 2.3.1	Multiplex PCR primers for the generation of *SLC22A1* amplicons used in SNaPshot™ genotyping.	**53**
Table 2.3.2	Multiplex PCR primers for the generation of *SLC22A2* amplicons used in SNaPshot™ genotyping.	**54**
Table 2.3.3	Multiplex PCR primers for the generation of *SLC22A3* amplicons used in SNaPshot™ genotyping.	**55**
Table 2.3.4	*SLC22A1* multiplex 1 single base extension primers.	**57**
Table 2.3.5	*SLC22A2* gene multiplex 1 single base extension primers for SNaPshot™ genotyping assay of selected SNPs.	**59**
Table 2.3.6	*SLC22A3* gene single base extension primers for SNaPshot™ genotyping assay of selected SNPs.	**61**
Table 2.4.1	Genotype and allele frequencies of OCT1 *(SLC22A1)* gene SNPs in 130 healthy CA individuals.	**66**
Table 2.4.2	Comparison of MAF of *SLC22A1* gene SNPs of the CA population to other ethnic groups.	**69**
Table 2.4.3	Haplotype structure defined by 20 SNPs in the *SLC22A1* gene in the CA population.	**71**
Table 2.4.4	Genotype and allele frequencies of 20 known *SLC22A2* coding SNPs 130 healthy Cape Admixed individuals.	**73**
Table 2.4.5	Comparison of MAF of selected *SLC22A2* SNPs in the local populations to other ethnic groups.	**75**
Table 2.4.6	Haplotype structure of *SLC22A2* gene in the Cape Admixed population as defined by the 20 selected loci.	**75**

Table 2.4.7	Genotype and allele frequencies of the OCT3 *(SLC22A3)* gene SNPs in 130 healthy Cape Admixed individuals.	**77**
Table 3.3.1	*SLCO1B1* multiplex PCR primers for the amplification of exons and flanking regions.	**93**
Table 3.3.2	Multiplex 1 of *SLCO1B1* single-base extension primers for SNaPshot™ mini-sequencing reactions.	**96**
Table 3.3.3	Multiplex 2 of *SLO1B1* single-base extension primers for SNaPshot™ mini-sequencing reactions.	**98**
Table 3.4.1	Genotype and allele frequencies of OATP1B1 *(SLCO1B1)* gene SNPs in 130 healthy CA individuals.	**99**
Table 3.4.2	Comparison of MAF of *SLC22A1* gene SNPs of the CA population to other ethnic groups	**102**
Table 3.4.3	Haplotype structure defined by 20 SNPs in the *SLCO1B1* gene in the CA population.	**105**
Table 4.4.1	PCR primer sequences for promoter region amplicon generation.	**117**
Table 4.4.2	Summary of SNPs identified with direct sequencing of the promoter regions of pharmaco-genes in the CA population.	**118**
Table 4.4.3	Comparison of MAF of selected promoter SNPs identified by direct sequencing in the CA population to other ethnic groups.	**121**
Table 4.4.4	Major haplotypes inferred from 18 promoter SNPs identified with direct sequencing.	**125**

Table 5.3.1 PCR primer sequences for cloning the promoter region in-frame. **132**

Table 5.5.1 Mean fluorescence measured in 23 replicates on green filter using a luminometer. **135**

TABLE OF CONTENTS

	Page
Title Page	**i**
Keywords	**ii**
List of Publications	**iii**
Abstract	**v**
Declaration	**vii**
Acknowledgements	**viii**
List of Abbreviations	**ix**
List of Figures	**xi**
List of Tables	**xiii**
Chapter 1 Literature Review	**1**
1.1. Introduction	**1**
1.2. Human genetic variation and its contribution to complex traits	**3**
1.3. Pharmacogenomics and Personalized Drug Therapy	**4**
1.4. Adverse Drug Reactions	**7**
1.5. Race, Ethnicity and Genetic Ancestry in Biomedical Research	**8**
1.6. Single Nucleotide Polymorphisms and Variability in Drug Response and Toxicity	**10**
1.7. Membrane Transporters	**10**
1.8. Solute Carrier Transporters	**12**
1.8.1. Organic Cation Transporters	**13**
1.8.1.1. Structure	**13**
1.8.1.2. Substrate Specificity	**15**
1.8.1.3. Tissue Distribution and Localization	**16**

1.8.1.4. Expression and Regulation of OCTs **17**

1.8.1.4.1. Short-Term Regulation **18**

1.8.1.4.2. Long-Term Regulation **19**

1.8.2. Multidrug and Toxin Extrusion 1 (MATE1) **21**

1.8.2.1. Structure **21**

1.8.2.2. Tissue Distribution, Membrane Localization and Substrate Specificity **22**

1.8.2.3. Regulation of MATEs **23**

1.8.3. Organic Anion Transporters **24**

1.8.3.1. Structure and Function of the OATPs **24**

1.8.3.2. OATP1B **25**

1.8.3.3. Pharmacogenetics of OATP1B1 **26**

1.9. Diseases influenced by OCT, OAT and MATE regulation **28**

1.9.1. Cholesterol homeostasis **28**

1.9.2. Cholesterol and Cancer **28**

1.9.3. Hyperlipidaemia and Diabetes **29**

1.9.4. Hyperlipidaemia and Hypertension **30**

1.10. Clinical association between OCT, OAT and MATE variant alleles and drug disposition, response and toxicity **31**

1.10.1 Oral Anti-Diabetic Drugs **31**

1.10.1. OCT and OAT SNPs as predictors of response to Anti-Cancer Drugs **34**

1.10.2. HMG-CoA Reductase inhibitors (Statins) **38**

1.11. OCTs and MATEs in drug-drug interactions **39**

1.12. Genomic Diversity and Personalized Medicine: The African Perspective **40**

1.13. Summary and Main Objectives of the Project **41**

Chapter 2 Genetic Polymorphisms and Haplotype Structure of *SLC22A* Genes in the Cape Admixed Population. **46**

Chapter 3 Assessment of Genetic Variations within the *SLCO1B1* Gene of the Cape Admixed Population. **89**

Chapter 4 Identification of Genetic Variations within Pharmaco-gene Promoters in the Cape Admixed Population . **112**

Chapter 5 Cloning of the Polymorphic *SLC22A2* Promoter Region and Development of Promoter Expression Assay. **127**

Chapter 6 Summary and Future Perspectives **139**

References **146**

CHAPTER 1

1. LITERATURE REVIEW

1.1. Introduction

Inter-individual differences in the clinical efficacy and the toxicity of medication are common amongst patients (Kalow, 2006, Shastry, 2005). These inter-individual differences in drug response could be due to age, sex, body weight, nutrition, organ function, infections, co-medication, environmental factors, and genetic variation (Sadee and Dai, 2005, Shastry, 2005). In general, genetic factors are estimated to account for 15-20% of inter-individual variations in drug disposition and responses (Choi and Song, 2008a).

Adverse drug reactions (ADRs) are side effects experienced during drug therapy within the approved dosage and labelling recommendations for the specific drug (Daly, 2012). Severe ADRs are a significant clinical problem which may result in disabilities or permanent damage, congenital abnormalities or birth defects, hospitalizations, life-threatening events, and death (Daly, 2013). ADRs are amongst the leading causes of hospitalizations in the developed world, and the incidence of severe ADRs has been estimated at 6.2-6.7% in hospitalized patients (Bachtiar and Lee, 2013). However, in developing countries like South Africa ADRs are estimated to occur in 14% of hospitalized patients (Mehta et al., 2008, Warnich et al., 2011). Moreover, the incidence of fatal ADRs is estimated at between 0.15-0.3% in developed countries and may be five to ten times higher in developing countries (Bachtiar and Lee, 2013, Mehta et al., 2008). The economic impact of ADR-related hospitalizations has been estimated at $136 billion in the USA alone (Becquemont, 2009, Bond and Raehl, 2006).

CHAPTER 1

It has long been recognized that genetic variations in drug metabolizing enzymes underlie the inter-individual differences in drug response. However, polymorphisms in solute carrier transporters (SLCs) are increasingly being recognized as a factor accounting for inter-individual variation in drug response and being involved in the toxicity of drug treatment or predisposition to ADRs. These polymorphisms are attracting interest because SLCs are widely distributed in the epithelial membrane of the liver, kidney, and intestine and play an important role in the gastrointestinal absorption, biliary and renal uptake and excretion, and distribution to target sites of their substrate drugs.

Approximately 40% of therapeutic drugs are organic cations or weak bases at physiological pH and are substrates of organic cation transporters (OCTs) and multidrug and toxin extrusion (MATEs) transporters (Neuhoff et al., 2003). The transport of OCs is mediated by OCTs in an electrogenic, and independently of a sodium gradient, and by MATEs through an oppositely directed proton gradient (Koepsell et al., 2007b, Otsuka et al., 2005). Examples of clinically important drugs transported by OCTs and MATEs include the antidiabetic drugs metformin and phenformin, the antineoplastic drugs cisplatin and oxaliplatin, the anti-HIV drugs lamivudine and zalcitabine, and the histamine receptor antagonist cimetidine (Barendt and Wright, 2002, Busch et al., 1998, Ciarimboli et al., 2005b, Dresser et al., 2002, Kimura et al., 2005b, Jung et al., 2008).

Previous studies have shown that human OCTs and MATEs are highly polymorphic in ethnically diverse populations (Sakata et al., 2004, Shu et al., 2003, Kang et al., 2007a). A number of these variants have been associated with reduced effect of therapeutic drugs, example the antidiabetic metformin and the

antineoplastic imatinib. However, these aforementioned reduced-function genetic variants were however primarily found in studies with European participants and have not been consistently replicated for other ethnic groups (Chen et al., 2010b, Leabman et al., 2003a).

Although Africa and South Africa harbour a significant proportion of genomic diversity and have a significant disease burden, the impact of this genomic diversity on the inter-individual differences in drug response is however understudied (Coovadia et al., 2009, Hardy et al., 2008, Tishkoff et al., 2009). Furthermore, pharmcogenomic and pharmacogenetic research in Africa is in its infancy and has primarily focused on drug metabolizing enzymes (Hardy et al., 2008, Warnich et al., 2011). Thus, this review summarizes our current understanding about the structure, distribution, substrate specificity, physiological roles of OCTs and MATEs and to discuss the importance of these transporters in the pharmacokinetics and pharmacodynamics of clinically important cationic drugs.

1.2. Human genetic variation and its contribution to complex traits

Human genetic variants are typically referred to as either common or rare, to denote the frequency of the minor allele in the human population. These variants are classified as single nucleotide polymorphisms (SNPs), insertions-deletions, varying number of tandem repeats (VNTRs), inversions, and copy number variants (Brockmöller and Tzvetkov, 2008). SNPs are the most prevalent class of variants amongst individuals.

Currently, it is estimated that the human genome contains at least between 11 and 12 million SNPs (Brockmöller and Tzvetkov, 2008). Moreover, approximately 7

million of these SNPs occur at a minor allele frequency (MAF) greater than 5% and the remaining at a MAF between 1 and 5%. The current opinion is that African populations harbour more genetic variation than other populations (Hardy et al., 2008, Tishkoff et al., 2009). This view is supported when considering the fact that the Yoruban genome has 1.25 fold more single base variants than the Caucasian genomes and that a greater percentage is novel, which is reflective of the overall increased amount of genome diversity in individuals of African origin (Frazer et al., 2009).

Variation in the human genome has a wide variety of medical and health implications. The current belief is that the knowledge acquired through human genetic studies will have a major impact on medical sciences, and that initially our increased understanding of the molecular pathways/mechanisms involved in disease will provide new potential drug targets (Frazer et al., 2009, Séguin et al., 2008, Daar and Singer, 2005a). Subsequently, the expectation is that this increased understanding will equip us to predict disease susceptibility, to classify diseases in sub-phenotypes from genotypic information, and to improve treatment and expand the use of pharmacogenomics (Frazer et al., 2009).

1.3. Pharmacogenomics and Personalized Drug Therapy

Pharmacogenetics is not a new discipline itself but has been around for approximately 50 years (Kalow, 2006). However, advances in genomics, especially in methodology, have given rise to pharmacogenomics, improving our ability to identify the genetic causes of specific diseases, search for novel drug targets, and to improve drug development (Daar and Singer, 2005a).

CHAPTER 1

Pharmacogenetics is defined as the discipline which uses the patient's genetic information of drug metabolizing enzymes, drug receptors, and drug transporters in order to develop and individualized drug therapy that will result in optimal choice and dose of the drugs in question (Holm, 2008). The science of pharmacogenetics originated from the analysis of a few rare and sometimes unexpectedly found extreme reactions (phenotypes) observed in some humans. These phenotypes were either inherited diseases or abnormal reactions to drugs or other environmental factors (Brockmöller and Tzvetkov, 2008). An important milestone in pharmacogenetics occurred when it became clear that drug effects tended to differ not only between individuals, but also between human populations (Kalow, 2006).

Pharmacogenomics, on the other hand, is defined as 'The study of variations of DNA and RNA characteristics as related to drug response' (Bhathena and Spear, 2008). Moreover, pharmacogenomics is an approach that has evolved from pharmacogenetics and has become a new scope for the pharmaceutical and biomedical fields (Khoury et al., 2008). It is widely expected that pharmacogenomics will facilitate a trend toward improved patient outcomes by increasing our understanding at the molecular level of both the disease and treatment response (Bhathena and Spear, 2008, Eichelbaum et al., 2006). Moreover, the pharmacogenomics approach has already supplied researchers with a number of candidate genes and their translational ramifications on drug response in many complex states (McCarthy and Zeggini, 2007).

Inter-individual difference in the efficacy and the toxicity of medication is common amongst patients (Shastry, 2005). This difference in drug response could

be due to age, sex, body weight, nutrition, organ function, infections, co-medication, environmental factors, the dose-response curve of a drug (pharmacokinetics and pharmacodynamics), and genetic variation (Sadee and Dai, 2005, Shastry, 2005).

However, when treating individual patients, the focus must be shifted from populations, ethnicities or races to the inherent genetic individuality that results from mosaics of variable haplotypes (Suarez-Kurtz, 2008b). Knowledge of an individual's genetic variability in drug response is, therefore, clinically and economically important. This type of genetic profiling provides benefits for future medical care by predicting drug response or assisting in the development of DNA-based tests. Thus, pharmacogenetics and pharmacogenomics are two recent developments to investigate inter-individual variations in drug response (Shastry, 2005).

While the initial focus of pharmacogenetics was on drug metabolizing pathways (pharmacokinetics), the focus of pharmacogenomics is on the genetic basis of the individual variation in drug efficacy and toxicity (pharmacodynamics) (Kalow, 2006, Urban, 2010). The assumption or expectation of both pharmacogenetics and pharmacogenomics is the ability to deliver "personalized medicine", a broad and rapidly advancing field of healthcare that is informed by each patient's unique, clinical, genetic, genomic, and environmental information (Holm, 2008, Limdi and Veenstra, 2010). That is, personalized medicine can be used to refine the definition of disease, identify disease subtypes, and ultimately define biomarkers capable of discriminating between the patients most likely to benefit from a

specific treatment and those unlikely to respond or likely to experience adverse events (Reitman and Schadt, 2007).

There are many challenges that must be overcome to apply rapidly accumulating genomic information to understand variable drug responses. These include, defining candidate genes and pathways; relating disease genes to drug response genes; precisely defining drug response phenotypes; and addressing analytic, ethical, and technological issues involved in the generation and management of large drug response sets (Roden et al., 2006). One of the impediments to the use of pharmacogenomics testing is the fact that some prescribing decisions must be made emergently, necessitating the availability of pre-emptive genotype results (Relling et al., 2010). Furthermore, various pharmacogenomic associated studies have not been reproduced and confirmed. In addition, the Genome Wide Association Studies (GWAS) that have been performed have been restricted primarily to populations of European descent, mostly because biomedical research funding is highest in the United States and Western Europe, where European ancestral populations make up the majority (Urban, 2010). Furthermore, a great deal of education for the public and healthcare professionals in the area is necessary before gaining overall acceptance (Avery et al, 2009).

1.4. Adverse Drug Reactions

Adverse drug reactions (ADRs) is defined as a response to a drug which is noxious and unintended, and occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function (WHO, 2004). The undesirable effects of the drug may lead to any of the following: death or a life-threatening event, hospitalization,

disability or permanent damage, congenital abnormality or birth defect (FDA, 2011).

From a clinical perspective, ADRs can be broadly classified as either Type A, which are dose-dependent, or Type B (idiosyncratic), where the reaction is not predictable from normal drug pharmacology and is generally dose-independent (Daly, 2013). Although Type A ADRs are more common, they are frequently mild and often self-limited. On the other hand, Type B ADRs are less common, but are often more severe and are more likely to result in serious morbidity or even mortality.

ADRs are implicated in a notable number of hospitalizations, and fatal ADRs are amongst the leading causes of death in developed nations (Sim and Ingelman-Sundberg, 2011, Wester et al., 2008). The incidence of severe ADRs, in the United States, has been estimated at between 6.2-6.7% in hospitalized patients and the incidence of fatal ADRs is estimated to be between 0.15-0.3%. In South Africa, on the other hand, ADRs are reported to occur in 14% of hospitalized patients with a five to ten times higher fatality rate (Mehta et al., 2008). In recent years, the economic cost of ADR-related hospitalizations has reached $136 billion in the USA alone (Becquemont, 2009, Bond and Raehl, 2006).

Genetic susceptibility is an important feature of serious ADRs and there is considerable interest in the possibility that development of genetic tests to identify all those at risk of adverse events prior to prescription might lead to valuable drugs being retained (Daly, 2013). In order to achieve this goal consideration must be given to the fact that population differences exist in drug response, including susceptibility to ADRs, and that these differences are in part due to genetic

polymorphisms (Bachtiar and Lee, 2013). Genetic variation frequencies differ among different ethnicities, which may be associated with variation of susceptibility to ADRs among different populations.

1.5. Race, Ethnicity and Genetic Ancestry in Biomedical Research

The problem of race in scientific research is not a new one, and the issue seems to perpetually reappear and remain fundamentally unresolved (Caulfield et al., 2009). However, it is a known fact that specific monogenic diseases such as sickle cell anaemia, Tay-Sachs, and cystic fibrosis for example, differ between populations (Via et al., 2009b). In order to use genomic knowledge to develop drugs and to improve health, we need to consider ethnical differences in different populations (Shastry, 2005). There exist inter-ethnical differences in polymorphisms of genes encoding drug metabolizing enzymes, transporters and disease-associated proteins (Bachtiar and Lee, 2013). Many gene variants differ in frequency between populations or subpopulations, but this is often merely due to random fluctuations (called genetic drift) and has no true biological meaning (Urban, 2010). With the availability of genetic ancestry estimates it is believed that admixed populations represent a valuable opportunity to study complex disease and drug response (Via et al., 2009b). Since admixed populations share varying proportions of different ancestral populations their genetic complexity can potentially complicate biomedical research. On the other hand, precisely because of this complexity, admixed populations can also provide unique opportunity to disentangle the clinical, social, environmental, and genetic underpinnings of population differences in health outcomes (Suarez-Kurtz, 2008b).

The accumulated data reveal that allele, genotype and haplotype frequency of polymorphisms of pharmacological relevant genes (pharmaco-genes) may differ significantly among populations categorized by race, ethnicity, or continental origin (Suarez-Kurtz, 2008b). It is widely accepted that population based pharmacogenetics studies can help in establishing baseline frequency distributions of SNPs of genes important in drug metabolism and/or transport. Therefore, recognition of interethnic differences in drug response might be useful in the establishment of public health policies, the design and interpretation of clinical drug trials, and possibly to guide clinicians to prospectively evaluate those patients with the greatest possibility of expressing a variant genotype which may be associated with inter-individual variation in drug response, efficacy and toxicity (Matimba et al., 2008, Suarez-Kurtz, 2008b).

1.6. Single Nucleotide Polymorphisms and Variability in Drug Response and Toxicity

Inter-individual variability in drug response and toxicity is a significant clinical and public health problem. This variation can be due to genetic, environmental, physiological, and pathophysiological factors (Choi and Song, 2008a, Giacomini et al., 2010).

A considerable body of evidence currently exists that suggests that single nucleotide polymorphisms (SNPs) in genes encoding drug metabolizing enzymes, enzymes involved in DNA biosynthesis and repair, and drug transporters might determine drug efficacy and toxicity (Shastry, 2005). Many drug metabolizing enzymes and drug transport proteins have consistently replicated associations between genetic variants and the clinical pharmacokinetics of at least one drug

(Bhathena and Spear, 2008). Genetic polymorphisms in membrane transporter genes are increasingly been recognized as a possible mechanism for explaining variation in drug response (Leabman et al., 2003a, Shu et al., 2007).

1.7. Membrane Transporters

Membrane transporters are specialized integral proteins that span cell membrane bilayers and play a critical role in the translocation of chemicals into and out of cells using active and passive mechanisms (Klaassen and Aleksunes, 2010). They are responsible for maintaining cellular and organismal homeostasis by importing nutrients essential for cellular respiration and exporting metabolic waste products and xenobiotics (Leabman et al., 2003a). These transporters are located in the epithelial membrane of the liver, kidney, intestine, and target organs and are now widely acknowledged as important determinants governing drug absorption, excretion, and, in many cases, extent of drug entry into target organs (Choi and Song, 2008a, DeGorter et al., 2012).

Inter-individual variation in transporter activity can arise from numerous factors, including genetic heterogeneity, certain disease processes, concomitant medications, and herbal and dietary constituents that may inhibit or induce transporter expression or activity (DeGorter et al., 2012, Giacomini et al., 2010). Numerous studies have suggested that membrane transporters play a part *in vivo* in drug disposition, therapeutic efficacy, and adverse drug reactions. Moreover, genetic polymorphisms in membrane transporter genes have increasingly been recognized as a possible mechanism accounting for variation in drug response (Leabman et al., 2003a, Shu et al., 2007). In general, genetic factors are estimated to account for 15-30% of inter-individual variations in drug disposition and

responses, however for certain drugs, this estimate can be as high as 95% (Choi and Song, 2008a, Eichelbaum et al., 2006, Evans and Relling, 2004).

During the last decade, a greater focus has been given to impact of genetic variations in membrane transporters on the pharmacokinetics and toxicity of numerous therapeutic drugs (Hediger et al., 2004). However, while the majority of transporter-related pharmacogenomic research has been in regards to classic genes encoding the outward-directed ATP-binding cassette (ABC) transporters, more studies have been conducted in recent years evaluating genes encoding solute carrier (SLC) transporters that mediate the cellular uptake, distribution and elimination of clinically important drugs (Franke et al., 2010).

1.8. Solute Carrier Transporters

The solute carrier transporter (SLC) superfamily is a large family of membrane-bound proteins that share 2025% of sequence homology (Hediger et al., 2004). This major facilitator family consists of more than 300 members grouped into 51 classes. SLC transporters typically use secondary and tertiary active transport to move chemicals over biological membranes (Klaassen and Aleksunes, 2010). They are transmembrane proteins which typically have a predicted membrane topology that consists of 12 αhelical transmembrane helices (TMHs), an intracellular N-terminus, a large glycosylated extracellular loop between TMHs 1 and 2, a large intracellular loop with phosphorylation sites between TMHs 6 and 7, and an intracellular C-terminus (Koepsell et al., 2007b). SLCs are expressed in most tissues. However, these proteins are expressed most abundantly in the liver, kidney, and intestine where they are either located at the basolateral or apical plasma membranes of polarized cells (Wojtal et al., 2009). Today it is known that

members of the SLC family are involved in the facilitated transport of a variety of substances including drugs, environmental toxins, xenobiotics, and endogenous metabolites across plasma membranes (Hediger et al., 2004, Koepsell et al., 2007b). Moreover, these SLC transporters play a critical role in the absorption and excretion of drugs in the kidneys, liver, and intestine, thus, influencing the pharmacodynamic and pharmacokinetic characteristics of these drugs (Meier et al., 2007b).

The SLC family can be divided into three subgroups based on substrate specificity and function: organic cation transporters (OCTs), the organic cation/zwitterion transporters (OCTNs), and the organic anion transporters (OATs) (Koepsell et al., 2007b). The OCT subgroup contains three subtypes of facilitated transporters called hOCT1 (encoded by the *SLC22A1* gene), OCT2 (*SLC22A2*), and OCT3 (*SLC22A3*). The genes encoding the three OCT isoforms are clustered together on the long arm of chromosome 6 (Tzvetkov et al., 2009, Koehler et al., 1997a). Based on their substrate properties and tissue distributions, hOCT1, hOCT2, and hOCT3 are thought to play important roles in the biliary and renal excretion of their substrates and the distribution of organic cationic drugs in the liver, kidney, heart, and brain (Jonker and Schinkel, 2004, Koepsell et al., 2007b). Moreover, there are several members of the SLC family for which the substrate specificity and/or function have not been elucidated yet.

1.8.1. Organic Cation Transporters

1.8.1.1. Structure

Members of the OCT family are highly conserved among species and generally 550-560 amino acids in length and share common structural features, including a

characteristic membrane topology, depicted in **Figures 1.1** and **1.2,** of 12 putative transmembrane spanning αhelices (TMHs), intracellular COOH and NH_2 termini, an intracellular loop with phosphorylation sites between the sixth and the seventh TMHs and a large extracellular loop between the first and second TMHs containing glycosylation sites (Burckhardt and Wolff, 2000, Ciarimboli, 2008, Koepsell et al., 2007b).

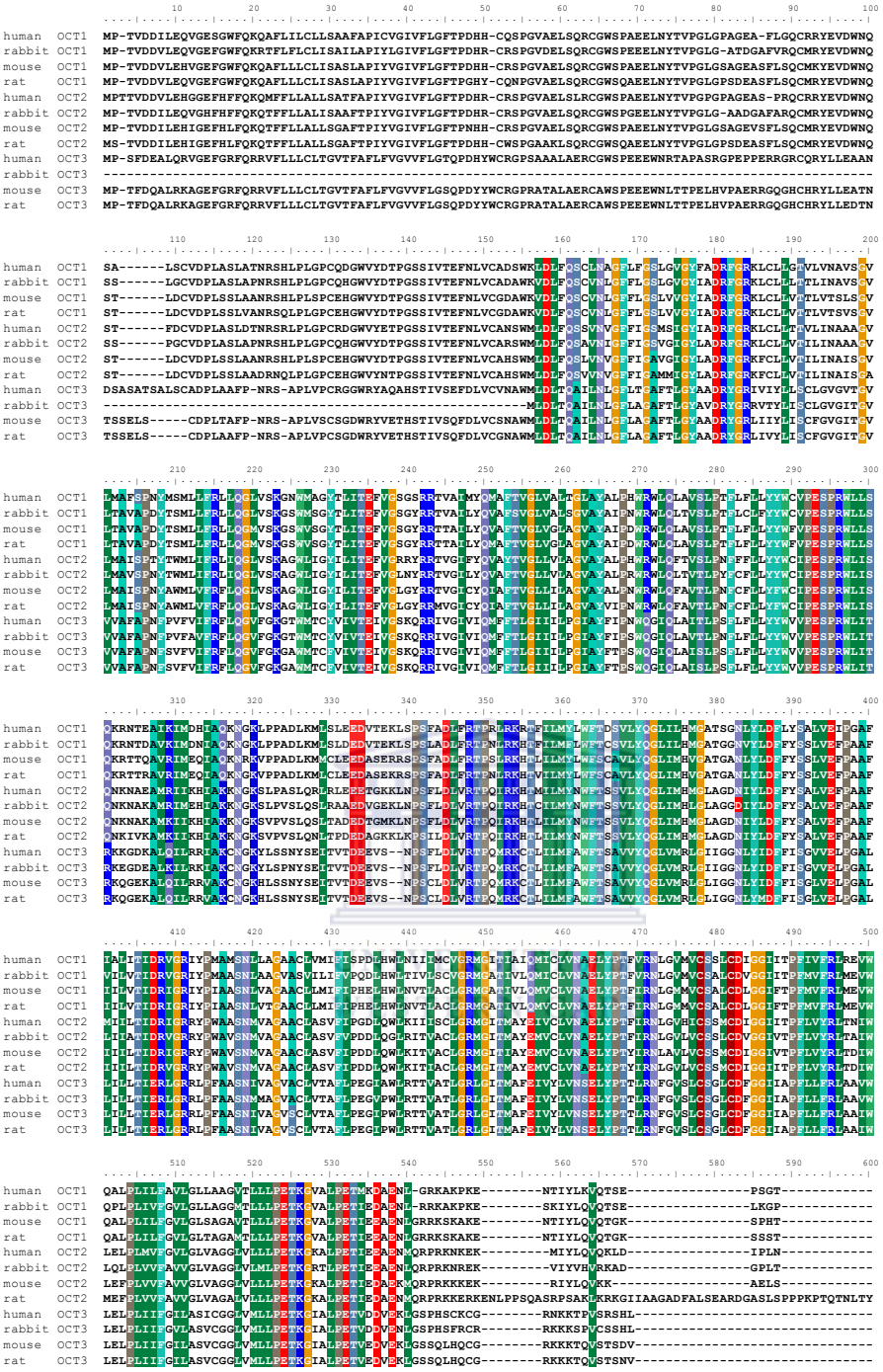


Figure 1.1. Multiple protein sequence alignment of OCTs from four animal species showing a high degree of evolutionary conservation.

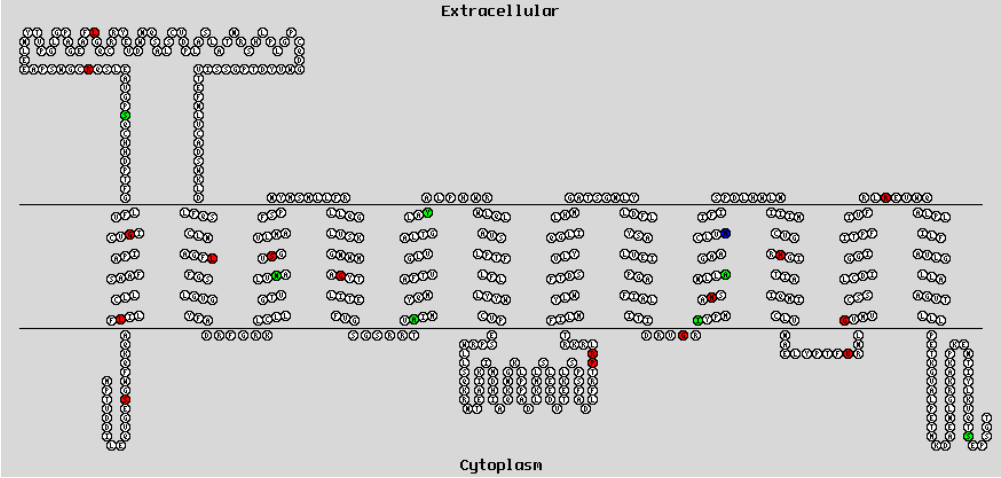


Figure 1.2. Predicted membrane topology of OCTs as represented by human OCT1 (UCSF Protein Membrane Transporter database, 2015)

1.8.1.2. Substrate Specificity

OCTs are defined as polyspecific transporters and function as uniporters that play a role in facilitated diffusion in either direction and are involved in translocation of organic cations, endogenous amines, therapeutic drugs and cationic xenobiotics with different molecular structures (Jonker and Schinkel, 2004, Koepsell et al., 2007b). There is extensive overlap of substrate and inhibitor specificities among hOCT13 from different species. Oct1/hOCT1 orthologs from four species (rat, mouse, rabbit, and human) all transport tetraethylammonium. However, the affinity and transport rates differ between the four species. Oct1/hOCT1 substrates include pharmaceuticals such as the antidiabetic drug metformin (Kimura et al., 2005a, Wang et al., 2002), the antiviral drugs acyclovir and zalcitabine (Jung et al., 2008, Takeda et al., 2002), the antineoplastic cisplatin (Ciarimboli et al., 2005b, Ciarimboli et al., 2010), the N-methyl-D-aspartate-receptor antagonist memantine, and the histamine H_2 receptor antagonist ranitidine. In addition, OCTs are also responsible for the transport of biogenic amine neurotransmitters **(Figure**

1.3) such as dopamine, epinephrine, norepinephrine, and histamine (Klaassen and Aleksunes, 2010).

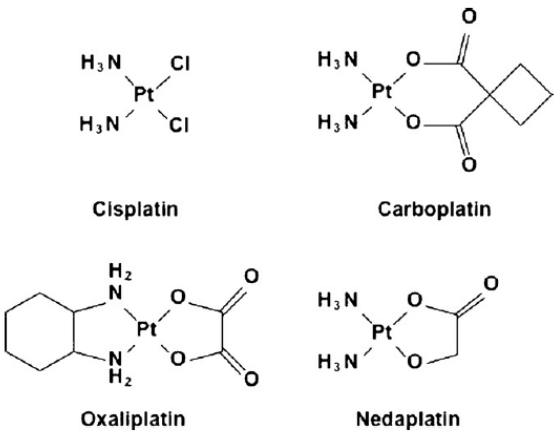


Figure 1.3. Chemical structures of 4 platinum agents which are substrates of hOCTs and hMATEs (Yokoo et al., 2007).

1.8.1.3. Tissue Distribution and Localization

The tissue distribution of the OCT subgroup is quite distinctive, with hOCT1 being primarily expressed in the basolateral or sinusoidal membrane of hepatocytes and also being present in the epithelial membrane of the intestine at low levels (Gorboulev et al., 1997a, Zhang et al., 1997). Thus, hOCT1 is thought to play a fundamental role in the uptake of substrates into the hepatocytes. On the other hand, hOCT2 is predominantly expressed at the basolateral membrane of the proximal renal tubules and facilitates uptake of substrates from the circulation into renal epithelial cells (Gorboulev et al., 1997a, Motohashi et al., 2002). hOCT3, on the other hand, shows a widespread tissue distribution, including the brain, heart, skeletal muscle, blood vessels, placenta, and liver (Koepsell et al., 2007b).

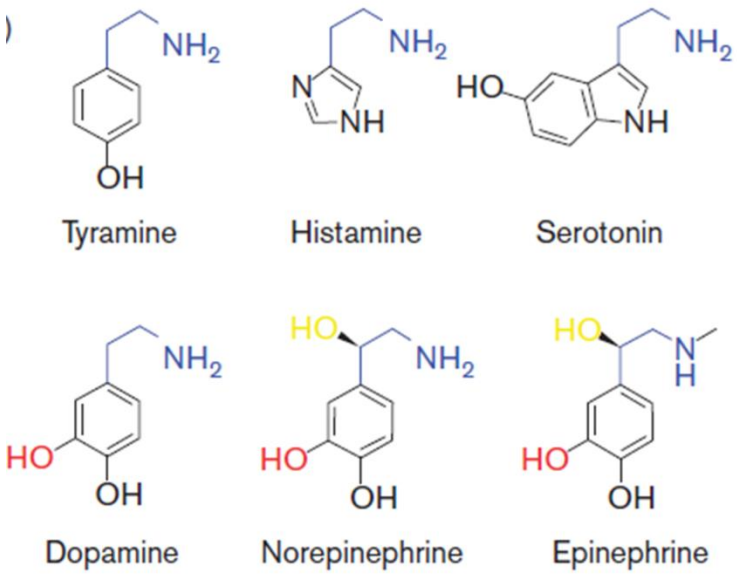


Figure 1.4. Chemical structures of selected bio-amine substrates of hOCTs (Chen et al., 2010a).

1.8.1.4. Expression and Regulation of OCTs

The regulation of OCTs has great physiological and even clinical importance because they can ultimately change the mRNA or protein levels of OCTs, and as a result, alter the absorption, secretion and tissue distribution of endogenous metabolites, drugs, and xenobiotics (Choi and Song, 2008a, Ciarimboli et al., 2005a). These regulatory mechanisms are important because stimulation of OCT expression can accelerate detoxification, whereas inhibition can prolong exposure of the body to dangerous substances. However, the regulation of OCTS is complex and may occur at the transcription, message stability, translation, and various posttranslational modification levels (Koepsell et al., 2007).

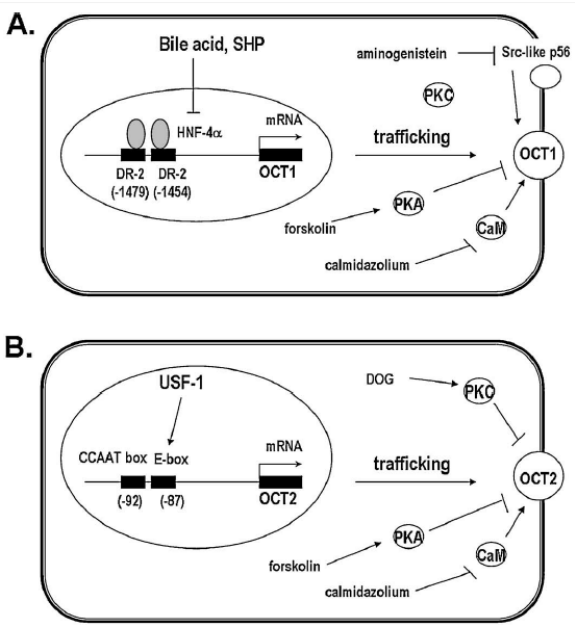


Figure 1.5. Schematic representation of transcriptional and posttranslational regulation of hOCT1 (A) and hOCT2 (B) (Choi and Song, 2008a).

1.8.1.4.1. Short-Term Regulation

Mechanisms of posttranslational activation of hOCT1 by its phosphorylation status have been proposed (Ciarimboli et al., 2004, Ciarimboli and Schlatter, 2005). There are multiple potential phosphorylation sites that are conserved among OCTs, which provide target sequences for functional regulation by kinases and phosphatases. hOCT1 and hOCT2 share common regulatory mechanisms involving protein kinase A (PKA) and calmodulin (CAM). hOCT1 is activated by Src-like $p53^{lck}$ tyrosine kinase (Ciarimboli et al., 2004). However, PKC activation decreases the affinity of hOCT1 for prototypical substrates (Ciarimboli and Schlatter, 2005). Expression levels of hOCT1 were unchanged by a PKA activator, whereas OCT2 was downregulated by the PKC activator 1,2diocanoylsnglycerol (DOG).

Çetinkaya *et al.* (2003) demonstrated that hOCT2 is inhibited by PKA and phosphatidylinositol 3kinase (PI3K) and activated by a CAM-dependent signalling pathway, probably through a change in substrate affinity (Cetinkaya et al., 2003). Furthermore, in a subsequent study Biermann *et al.* (2006) showed that inhibition of the Ca^{2+}/CAM complex by calmidazolium causes changes in transport capacity due to reduced hOCT2 trafficking/localization to the plasma membrane (Biermann et al., 2006).

1.8.1.4.2. Long-Term Regulation

Transcription factors may be responsible for the constitutive expression of *SLC22A1*. In a study by Saborowski *et al.* (2006) it was demonstrated that transcriptional activation of *SLC22A1* can be mediated by the binding of hepatocyte nuclear factor 4α (HNF4α) to DNA response elements (DR2) adjacent to the gene and suppressed by bile acids via the bile acid-inducible transcriptional repressor, small heterodimer partner (SHP) (Saborowski et al., 2006). Furthermore, Rulcova *et al.* (2013) in a recent study showed that *SLC22A1* expression is indirectly induced by glucocorticoid activation through the upregulation of HNF4α in primary hepatocytes (Rulcova et al., 2013). Moreover, Asaka *et al*. (2007) demonstrated that basal transcription of *SLC22A2* was stimulated by binding of the ubiquitously expressed and constitutively active upstream stimulating factor (USF) 1 to the proximal promoter region (Asaka et al., 2007). In a recent study O'Brien et al showed through electrophoretic mobility shift and chromatin immunoprecipitation assays that the expression of hOCT1 is regulated by HNF1 through binding to an evolutionary conserved region in intron 1 of *SLC22A1* (O'Brien et al., 2013).Epigenetic gene silencing may also provide a

mechanism of organic cation transporter gene regulation. Recent studies have shown that DNA methylation of *SLC22A1* in hepatocellular carcinoma (HCC) (Schaeffeler et al., 2011) and *SLC22A3* in prostate cancer (Chen et al., 2013) is associated with reduced expression of these genes.

Figure 1.6. Multiple protein sequence alignment of MATE protein sequences from four different animal species.

1.8.2. Multidrug and Toxin Extrusion 1 (MATE1)

1.8.2.1. Structure

hMATE1 and the kidney-specific hMATE2K are orthologs of the multidrug and toxin extrusion (MATE) family of bacteria. hMATE1 is encoded by the *SLC47A1* gene located at 17p11.2 with gene organization as depicted in **Figure 1.7** (Otsuka et al., 2005, Terada and Inui, 2008). MATEs are highly conserved among species as shown in **Figure 1.6** with human, mouse, rat, and rabbit MATE1 being 570, 532, 566, and 568 amino acid residues in length, respectively (Otsuka et al., 2005, Terada et al., 2006). While the prokaryotic, fungal, and plant MATE family members share a predicted membrane topology of 12 TMHs, Zhang and Wright's study has shown that hMATE1and hMATE2K appear to have an additional COOH-terminal helix (Zhang and Wright, 2009).

Initial studies using rat renal brush-border membranes vesicles have indicated that cysteine and histidine residues are critical for H+/organic cation antiporter activity (Hori et al., 1987, Hori et al., 1989). Subsequently, Matsumoto *et al.* (2009) showed that when the conserved Glu273, Glu278, Glu300, and Glu389 residues of hMATE1 were substituted with alanine or aspartate transport activity was reduced, suggesting an important role in the transport function of the MATE family (Matsumoto et al., 2008).

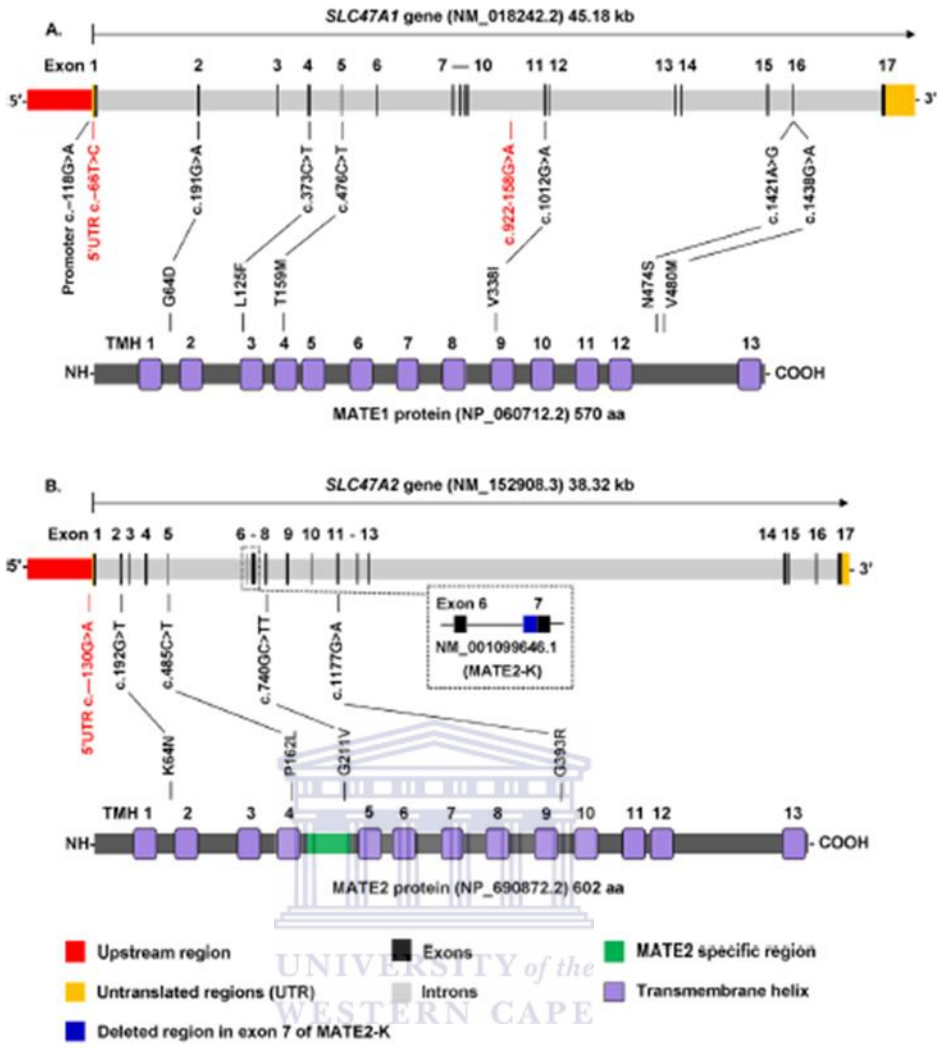


Figure 1.7. A schematic representation of the gene organization and the primary protein structure of human (A) *SLC47A1* (hMATE1) and (B) *SLC47A2* (hMATE2K). The diagram depicts exons (numbered 117) and introns of both genes together with the arrangement of transmembrane helices (TMHs) (numbered 113) of encoded proteins (Staud et al., 2013).

1.8.2.2. Tissue Distribution, Membrane Localization and Substrate Specificity

hMATE1 is predominantly expressed at the luminal membranes of the renal proximal tubules (kidney) and the bile canaliculi (liver) and mediates the secretion of organic cations by using an oppositely directed H^+ gradient as a driving force (Masuda et al., 2006, Otsuka et al., 2005, Tsuda et al., 2009). On the hand,

hMATE2K is primarily expressed in the brush-border membrane of renal proximal tubules (Masuda et al., 2006).

hMATE1 and hMATE2K mediates the H^+-coupled electroneutral exchange of tetraethylammonium (TEA) and 1methyl4phenylpyridinium (MMP^+), two prototypical organic cation substrates of renal and hepatic H^+-coupled organic cation antiporters (Koepsell et al., 2007b, Tanihara et al., 2007). In addition, MATEs are also involved in the transport of clinically important drugs such as metformin, cimetidine, and procainamide (Tanihara et al., 2007). K_m values of cationic drugs for hMATE1 and hMATE2K are similar and higher than the plasma concentrations in clinical use. As a key element in the renal (and hepatic) secretion of cationic drugs, the human MATEs are likely targets for unwanted drug-drug interactions, as well as principal arbiters of the pharmacodynamics and pharmacokinetics of many clinically important agents (Zhang et al., 2012). Although MATEs recognize substrates similar to the OCT family, MATEs can transport zwitterions and anionic compounds in addition to cationic drugs. In spite of having overlapping substrate spectra, MATE1 and MATE2-K do differ in substrate specificity and affinity.

1.8.2.3. Regulation of MATEs

In contrast to OCTs, little information is available on the regulation of MATEs. The proximal promoter region of human, murine, and rat *SLC47A1* genes lacked a canonical TATA-box but contained two conserved Sp1binding consensus sequences. Moreover, disruption of Sp1 binding through mutagenesis affected hMATE1 activity that lead to an approximate 50% reduction relative to the control.

In a study by Lickteig *et al* (2008) the pharmacological induction of MATEs in the liver by the activation of known transcription factors was investigated (Lickteig et al., 2008). Their study suggested that none of Aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane x receptor (PXR), peroxisome proliferator-activated receptor α (PPARα), and NFE2related factor 2 (NrF2) were able to alter hMATE1 or hMATE2 function.

1.8.3. Organic Anion Transporters

Oatp1a1 was first member of OATP/SLCO superfamily to be isolated from rats (Jacquemin et al., 1994). Subsequently, Oatp2a1 was isolated by Kinai et al (1995). The first human superfamily member (OATP1A2) was isolated and described by (Kullak-Ublick et al., 1995). Subsequently, further OATPs have been identified and characterized from various species using homology screening by either hybridization experiments or *in silico* techniques. OATPs have been identified in the intestine, liver, kidney, lung, testis, placenta and blood-brain barrier. Their substrates include endogenous substances such as bile salts, steroid conjugates and thyroid hormones, as well as several xenobiotics such as statins (Seithel et al., 2008).

1.8.3.1. Structure and Function of the OATPs

OATPs are comprised of 12 transmembrane domain (TMD) proteins with common structural features. This includes a large extracellular loop between TMD 9 and 10, *N*-glycosylation sites in extracellular loops 2, 3 and 5, and the consensus superfamily signature; DXRW(I,V0GAWWXG9F,L)L at the border between extracellular loop 3 and TMD 6 (Hagenbuch and Meier, 2003).

OATPs are sodium-independent uptake transporters that mediate the influx of a variety of amphipathic compounds. Their transport mechanism is electroneutral exchange, coupling the cellular uptake of organic compounds with the efflux of neutralizing anions such as bicarbonate, glutathione and glutathione-S-conjugates (Li et al., 1998, Satlin et al., 1997). The majority of OATPs transport a wide assortment of amphipathic organic compounds (Bossuyt et al., 1996). Oatp1a1 was initially isolated in rat liver as a sodium-independent bromosulfophthalein and taurocholate uptake system (Jacquemin et al., 1994).

Thereafter, comprehensive functional characterization, in different experimental systems, indicated that it can facilitate the transport of a wide variety of amphipathic organic compounds including bile salts, steroid hormones, thyroid hormones, organic cations including *N*-(4,4azo*n*pentyl)21deoxyajmalinium and numerous drug compounds. The included drugs are BQ123, CRC220, [D-penicillamine 2,5] (DPDPE) & deltorphin II, ACE inhibitors enalapril & temocaprilat, the HMG-CoA reductase inhibitor pravastatin and fexofenadine, an antihistamine (Hagenbuch and Meier, 2003, Hagenbuch and Meier, 2004, Meier et al., 2007a). The majority of member of the OATP1A and OATP1B subfamilies have been shown to exhibit similar broad, as well as partially overlapping, substrate specificities. This indicates that they may play a vital role, in conjunction with P-glycoproteins and multidrug resistance proteins, in drug absorption and disposition (Hagenbuch and Meier, 2004).

1.8.3.2. OATP1B

OATP1B is subdivided into two human members, OATP1B1 and OATP1B3. OATP1B1 cloned from human liver cells (Abe et al., 2001) encodes a 691 amino

acid glycoprotein which share 80% sequence homology with OATP1B3. However, it shares only 65% sequence homology with rat Oatp1b2 (Hagenbuch and Meier, 2003). OATP1B1 is expressed at the basolateral plasma membrane of hepatocytes (Tamai et al. 2000; Abe et al. 1999) and has a total mass of 84kDa, which is reduced to 54kDa after de-glycosylation (Konig et al., 2000). Since it is primarily expressed in the human liver it is suggested that OATP1B1 plays a critical role in the hepatic clearance of albumin-bound amphipathic compounds (Hagenbuch and Meier, 2004). This includes bile salts, conjugated and unconjugated bilirubin, BSP, steroid conjugates, thyroid hormones T4 and T3, eicosanoids, cyclic peptides, drugs including benzyl-penicillin, methotrexate, pravastatin and rifampicin as well as natural toxins such as mircosystin and phalloidin (Hagenbuch and Meier, 2003).

OATP1B3 was cloned from human liver and encodes a 702 amino acid glycoprotein with a molecular mass of 120kDa, which is reduced to 65kDa after de-glycosylation (Konig et al., 2000). OATP1B3 is exclusively expressed at the basolateral plasma membrane of hepatocytes. However, it has been shown to be expressed in several human cancer tissues, but the significance of this has yet to be investigated (Hagenbuch and Meier, 2004, Konig et al., 2000). OATP1B3 has similar broad substrate specificities as OATP1B1 transports bile salts, mono-glucuronosyl bilirubin, BSP, steroid conjugates, thyroid hormones T3 and T4, leukotriene C_4, linear and cyclic peptide, cardiac glycosides such as digoxin and ouabain, methotrexate, rifampicin and natural toxins such microcystin and phalloidin (Hagenbuch and Meier, 2003). However, OATP1B3 also exhibits unique transport capabilities, mediating transport of cholecystokinin 8 (CCK8),

deltorphin II and cardiac glycosides digoxin and ouabain (Konig et al., 2000, Kullak-Ublick et al., 2001).

1.8.3.3. Pharmacogenetics of OATP1B1

OATP1B1 is encoded by the SLCO1B1 gene, located on chromosome 12 (12p12). Numerous synonymous and nonsynonymous SNPs have been discovered on the SLCO1B1 gene. Many of these variants have been shown to affect transport function, both *in vitro* and *in vivo* (Iwai et al., 2004, Tirona et al., 2001). Furthermore, it has been recognized that most of these SNPs span the transmembrane domain or extracellular loop 5 of OATP1B1 (Iwai et al., 2004, Tirona et al., 2001). However, it is important to note that the effects of certain SLCO1B1 SNPs on transport function are substrate-specific (Tirona et al., 2001).

Within SLCO1B1 numerous nonsynonymous SNPs are prevalent. This includes RS4149056 and RS2306283, two of the most characterized SNPs in this gene, which produce amino acid changes Val174Ala and Asn130Asp respectively. However, when the effects of particular SNPs are evaluated it is important to take into account the underlying haplotype these SNPs are found in (Mwinyi et al., 2008). For example, the abovementioned SNPs are in linkage equilibrium and exist in varying haplotypes together. However, when the nucleotide Adenine (A) is present at RS2306283 and Thymine (T) is present at RS4149056 it is referred to as haplotype *1A or the reference haplotype (Table 1). Furthermore, SNPs in the promoter region of this gene can further sub-classify its haplotype into two distinct functional haplotypes (table 1) (Niemi et al., 2004).

Haplotypes *5 and *15 have been associated with a strikingly reduced uptake of multiple OATP1B1 substrates including oestrone3sulphate, oestradiol17βD-

glucuronide, atorvastatin, cerivastatin, pravastatin and rifampicin (Iwai et al., 2004, Kameyama et al., 2005, Nozawa et al., 2005, Tirona and Kim, 2005, Tirona et al., 2001). Additionally, RS4149056>C has been associated with increased plasma concentrations of many statins as well as fexofenadine and repaglinide (Chung et al., 2005, Lee et al., 2005, Mwinyi et al., 2008, Niemi et al., 2005a, Niemi et al., 2005b, Niemi et al., 2004, Nishizato et al., 2003).

It is important to note that these haplotype designations only refer to the SNPs in linkage disequilibrium. Other, less studied SNPs also occur in the SLCO1B1 gene which are potentially functional variants.

1.9. Diseases influenced by OCT, OAT and MATE regulation

1.9.1. Cholesterol homeostasis

Cholesterol is a major component of mammalian cell membranes. It is involved in controlling fluidity and maintaining the barrier between the cell and its environment. Furthermore, tissue growth, bile acid synthesis in the liver, vitamin D production in the skin and steroid hormone synthesis in the adrenal glands, ovaries and testes is also mediated by cholesterol (Brown and Goldstein, 1986). Cholesterol is transported in the circulatory system by lipoproteins, which are spherical particles with a hydrophobic core of esterified cholesterol and triglycerides surrounded by a hydrophilic shell of apolipoproteins (apos), phospholipids and unesterified cholesterol. Plasma lipoproteins can be characterised by their density into very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Lipoprotein receptors, specifically LDL receptors located on the surface of cells, bind LDL and transport cholesterol into cells via

receptor-mediated endocytosis where it will either be used immediately or stored as cytoplasmic cholesteryl ester droplets (Brown and Goldstein, 1983, Brown and Goldstein, 1986).

1.9.2. Cholesterol and Cancer

Recent discovery indicates that elevated glycolytic activity in various cancer types (Warburg effect) is a crucial feature of the cancer (Christofk et al., 2008, Fantin et al., 2006, Weinberg et al., 2010). Correspondingly, an increased cholesterol level in malignant cell transformation has long been debated. This phenomenon was first suggested in the early 20th century where xenografts were shown to have accelerated tumour growth rates following injection with cholesterol (Robertson and Burnett, 1913). However, research into this area has been lacking. Abnormal regulation of cholesterol homeostasis has been associated with numerous types of cancer. Several studies have established increased cholesterol levels in tumours as compared with normal tissues (Dessi et al., 1992, Kolanjiappan et al., 2003, Rudling and Collins, 1996, Schaffner, 1981, Yoshioka et al., 2000). Furthermore, low serum cholesterol levels have been associated with the presence of tumours in cancer patients. This suggests that cholesterol is accumulated in the tumour tissue) (Benn et al., 2011, Jacobs et al., 1992, Strasak et al., 2009). Multiple paths exist in cancer cells which result in increased intracellular cholesterol. This includes upregulation of 3hydroxy3methylglutayl CoA reductase (HGM-CoA) activity, loss of feedback inhibition of HMG-CoA by cholesterol, increased uptake of extracellular cholesterol via LDL receptor and decreased expression of the ATP-binding cassette transporter A1 (ABCA1) (Basso et al., 2005, Caruso et al., 2002, Graziani et al., 2002, Gregg et al., 1986, Hentosh et al., 2001, Ki et al., 2007, Moustafa et al., 2004, Notarnicola et al., 2004, Schimanski et al., 2010, Siperstein,

1995, Tatidis et al., 2002). Moreover, recent studies have shown that various statins, which block HMG-CoA activity and thereby inhibit cholesterol synthesis, reduce tumour growth in xenograft models (Huang et al., 2010, Kochuparambil et al., 2011).

1.9.3. Hyperlipidaemia and Diabetes

Diabetic patients suffer and increased risk of morbidity and mortality from cardiovascular disease (CVD) (Beckman et al. 2002). Patients with Type2 diabetes have an approximated two to fourfold increased risk of coronary artery disease (CAD). This is further compounded by the presence of such complications as diabetic nephropathy (Stephenson et al., 1995). Notably, the short term and longer term consequences for diabetic patients following myocardial infarction or stroke are generally higher than those of non-diabetic patients. Therefore, prevention of CAD in these high-risk patients is of vital importance (Yudkin, 1998). (Turner et al., 1998), showed that increased concentrations of LDL cholesterol, low levels of HDL cholesterol, hyperglycaemia, systolic hypertension and smoking are potentially modifiable risk factor for CAD in diabetic patients. Moreover, it has been established that low HDL cholesterol levels are more pathogenic in the presence of Type-2 diabetes. This is thought to be a result of the presence of small, dense LDL cholesterol particle and oxidation of glycated LDL cholesterol particles. Glycated LDL cholesterol particles are formed by the non-enzymatic reaction of glucose with apoproteins (Haffner, 1998). However, regardless of the importance of elevated LDL levels, the dyslipidaemia encountered in diabetic patients is more complex, interacting with numerous components in insulin resistance syndrome to enhance the risk of CVD through quantitative and qualitative changes (Krentz, 2003).

1.9.4. Hyperlipidaemia and Hypertension

It is estimated that up to 40% of hypertensive patients have high cholesterol levels (Hyperlipidaemia) and over 40% are obese (Criqui et al., 1980, Ostrander and Lamphiear, 1976). Hypertension and hyperlipidaemia are thought to be related as insulin resistance and saturate fat intake are common features in the pathogenesis of these diseases, as shown by epidemiological studies (Goode et al., 1995, Hopkins et al., 1996, Kannel et al., 1991). This suggests that hypertension and hyperlipidaemia act synergistically as cardiovascular risk factors.

1.10. Clinical association between OCT, OAT and MATE variant alleles and drug disposition, response and toxicity

1.10.1 Oral Anti-Diabetic Drugs

Type-2 diabetes mellitus is carbohydrate metabolism disorder. It manifests as hyperglycaemia as a result of defects in insulin secretion and insulin resistance. It is often related to symptoms of metabolic syndrome such as obesity, hyperlipidaemia, hypertension and endothelial dysfunction (Ryden et al., 2007). Oral antidiabetic therapies include metformin, sulfonylureas, thiazolidinediones, meglitinides, insulin, exenatide and dipeptidylpeptidase4 inhibitors (Edwards et al., 2008, Ryden et al., 2007). Patients with type-2 diabetes are often also treated with statins or fibrates, anti-hypertensives and acetylsalicylic acid (Ryden et al., 2007). However, since SLCO1B1 directly influences the meglitinide class of oral antidiabetic drugs, repaglinide will be focused on. Repaglinide is a short-acting meglitinide analogue that enhances glucose-stimulated insulin secretion from pancreatic beta cells, thereby reducing blood glucose concentrations (Dornhorst, 2001). Its mechanism of action is binding and closing the ATP-sensitive K^+

channels. Binding and closing the K^+ channel serves to depolarize the beta cell, allowing for the opening of voltage-gated Ca^{++} channels (Hatorp, 2002). This results in the influx of Ca^{++} ions which triggers the release of insulin by exocytosis. However, insulin release is dependent on the beta cell secretory capacity (Hatorp, 2002). The *SLCO1B1* RS4149056C polymorphism has been shown to directly affect repaglinide plasma levels (Kajosaari et al., 2005). This is particularly evident when atorvastatin is used in combination with repaglinide. It is suggested that the atorvastatin acts as an inhibitor, thus inhibiting OATP1B1mediated hepatic repaglinide uptake (Chung et al., 2005, Lee et al., 2005, Mwinyi et al., 2008, Niemi et al., 2004, Nishizato et al., 2003).

Metformin is a biguanide organic cationic (pKa 12.4) drug that is routinely prescribed as the preferred first-line therapeutic drug in the treatment of type-2 diabetes mellitus (Kirpichnikov et al., 2002, Nathan et al., 2009). It improves insulin sensitivity and thus decreases the insulin resistance that is prevalent in type-2 diabetes mellitus. Metformin is not metabolized and is excreted unchanged by active tubular secretion and glomerular filtration into the urine.

Although, the exact mechanistic pathway for metformin action is currently only partially understood, it is widely believed to activate adenosine monophosphate (AMP) activated protein kinase (AMPK) by inhibition of the mitochondrial respiratory chain (Owen et al., 2000, Zhou et al., 2001). Moreover, this activation of AMPK by metformin requires the phosphorylation of AMPK by a serine-threonine kinase, LKB1 (Shaw et al., 2005). This results in an increase in cellular AMP levels which in turn lead to insulin suppression of glucose production via gluconeogenesis and increased peripheral glucose uptake (Hawley et al., 2010, Hundal et al., 2000, Zhou et al., 2001). However, evidence exists which suggests

that metformin also exerts its metabolic effects via AMPK-independent mechanisms.

In addition to its favourable effect on hyperglycaemia, metformin also has other beneficial effects. Firstly, metformin is able to exert its glucose-lowering actions with a low risk of hypoglycaemia. Secondly, it reduces the likelihood of developing macrovascular (hypertension and atherosclerosis) and microvascular (neuropathy, retinopathy, and nephropathy) complications. Thirdly, it is weight neutral, that is, it does not affect body mass index (BMI) or decrease body weight in obese patients with or without diabetes.

However, approximately 30-40% of patients using metformin as an antidiabetic therapeutic experience adverse events such as diarrhoea and nausea; or a more serious but rare side effect, lactic acidosis (Reitman and Schadt, 2007, Takane et al., 2008, Wang et al., 2002). Moreover, approximately 38% of metformin users did not achieve acceptable control of fasting glucose levels and showed a variable glycaemic response (Reitman and Schadt, 2007). This variation in glycaemic response to metformin was attributed to the uptake of the drug either at the hepatic level or the elimination of the drug at the renal level. Furthermore, it was established that genetic variations in solute carrier transporter (*SLC*) genes, specifically hOCT1 (hepatic level) and hOCT2 (renal level), are involved in this varied response to the drug **(Figure 1.8)** (Kimura et al., 2005a, Wang et al., 2002). In addition, genetic variation in another SLC, hMATE1 transporter protein was also implicated in the glucose lowering effect of metformin (Becker et al., 2009b).

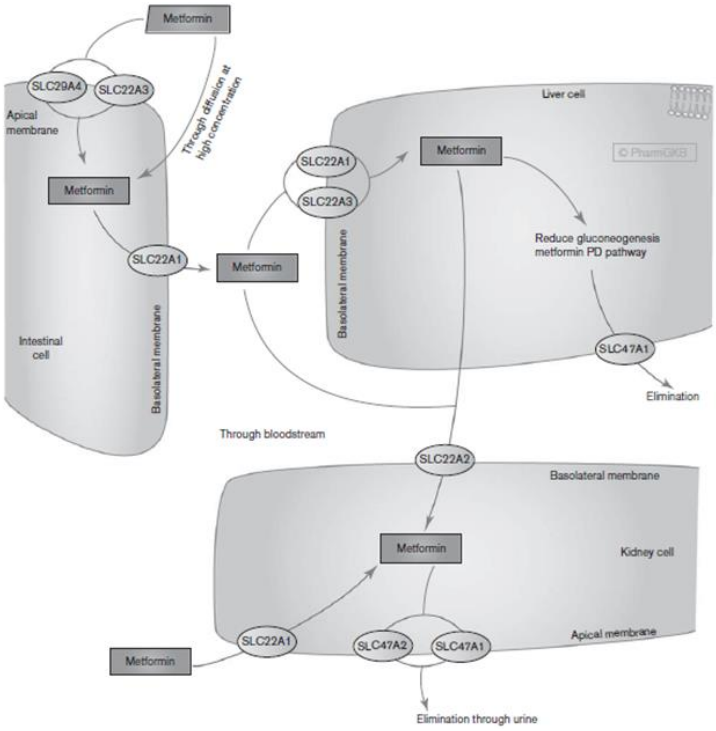


Figure 1.8. Pharmacokinetics pathway of metformin (Gong et al., 2012).

1.10.2. OCT and OAT SNPs as predictors of response to Anti-Cancer Drugs

OCTs may play an important role in the treatment of malignant tumours. For example, hOCT1 is responsible for the active uptake of the charged hydrophilic anticancer agents imatinib, cisplatin, oxaliplatin, picoplatin, irinotecan, and paclitaxel, thus contributing to the susceptibility of cancer cells to these antineoplastic drugs (Gupta et al., 2012). hOCT1 activity was reported to correlate well with the sensitivity of tyrosine kinase inhibitors (TKIs) such as imatinib in patients with chronic myeloid leukaemia (CML).

Yokoo *et al*. (2008) investigated whether hOCT3 was significantly involved in the oxaliplatin-induced cytotoxicity and accumulation of platinum in colorectal cancer (Yokoo et al., 2008). They found that *SLC22A3* expression was higher in colon and rectal cancerous tissues compared to normal tissues in Caucasian

patients. Moreover, they found that the cytotoxicity and accumulation of platinum caused by the treatment of oxaliplatin but not cisplatin depended on *SLC22A3* expression. Li *et al.* (2012) in a recent study investigated whether drug transporters played a role in determination of cisplatin resistance in cervical cancer cells (Li et al., 2012). They found that hOCT3 partially contributed to the sensitivity of adenocarcinoma cells to cisplatin cytotoxicity. Based on their data they suggested that downregulation of *SLC22A3* as a mechanism responsible for cisplatin accumulation in cervical adenocarcinoma cells. Expression of hOCT3 in kidney carcinoma cell lines increases chemo-sensitivity to the anti-neoplastics melphalan, irinotecan, and vincristine (Shnitsar et al., 2009).

In a recent study, Heise *et al.* (2012) investigated the impact of OCT expression on hepatocellular carcinoma (HCC) and patient survival (Heise et al., 2012). They found that downregulation of *SLC22A1* expression in HCC is associated with advance tumour stages and a worse patient survival rate. Downregulation of *SLC22A1* expression was also associated with tumour progression and reduced patient survival in human cholangiocellular carcinoma (CCA) (Lautem et al., 2013). Moreover, the downregulation of *SLC22A1* was significantly associated with advanced CCA stages. These findings could be important in future treatment strategies for these diseases.

Mohelnikova-Duchonova *et al.* (2013) investigated the association between the expression of solute carrier transporters and the prognosis of pancreatic cancer (Mohelnikova-Duchonova et al., 2013). They found that expression of some SLCs predicted the outcome of PDAC patients regardless of chemotherapy and that there is considerable variability in the expression in SLC transporters between

tumour and normal human pancreas tissues which may modify the outcomes of patients treated with nucleoside analogues and platinum containing regimens.

A common variant in 6q26q27 is associated with distal colon cancer in a Japanese population (Cui et al., 2011). However, Zhu *et al.* (2013) found that the genetic variant rs7758229 in 6q2627 was not associated with colorectal cancer risk in a Chinese population (Zhu et al., 2013). Genetic and functional analysis have implicate the *SLC22A3* gene together with 2 other genes in prostate cancer pathogenesis (Grisanzio et al., 2012).

Chronic myeloid leukaemia (CML) is a hematopoietic stem cell disorder, characterized by the presence of the Philadelphia (Ph) chromosome that results from a balanced reciprocal translocation between chromosomes 9 and 22 (Singh et al., 2012). Functionally, this translocation results in the formation of the *BCRABL* gene which is then translated into the BCRABL a protein with intrinsic tyrosine kinase activity that is critical to the development of CML (Rowly, 1973). Imatinib mesylate, a tyrosine kinase inhibitor (TKI), is now the first-line therapeutic for the treatment of chronic phase CML (White et al., 2010). However, 30-40% of patients with CML are resistant to imatinib treatment and do not achieve a complete cytogenic response (CCR) (Druker et al., 2006, Hochhaus et al., 2009).

This heterogeneity in imatinib response could be attributed to the presence of SNPs in the *SLC22A1* gene which codes for the hOCT1 transporter protein (Giannoudis et al., 2013). hOCT1 is an influx organic cation transporter that mediates the uptake of imatinib, a selective inhibitor of the oncogenic protein Bcr/Abl, into CML cells (Crossman et al., 2005, Thomas et al., 2004). In a recent study by Giannoudis *et al.* (2013), the effect of polymorphisms rs628031

(M408V) and rs35191146 (M420del) on imatinib uptake and clinical efficacy was investigated (Giannoudis et al., 2013). In CML cell lines transfected with the M420del variant and/or M408V variant, M420del significantly decreased imatinib uptake. However, this effect was countered if the M408V SNP was also present. Giannoudis *et al.* (2013) concluded that the only SNP associated with imatinib treatment outcome was M420del (rs35191146), with patients with the M420del genotype demonstrating an increased probability of imatinib treatment failure (Giannoudis et al., 2013).

However, the role of hOCT1 in the uptake of imatinib is controversial. In a recent study, Nies *et al.* (2014) challenged the role of hOCT1 in imatinib uptake, and showed through transport and inhibition studies that overexpression of functional hOCT1 did not lead to increased accumulation of imatinib (Nies et al., 2014). They concluded that cellular uptake of imatinib is independent of hOCT1 and as such hOCT1 is not a valid biomarker for imatinib resistance.

(Faber et al., 2003) first showed that anti-folate aminopterin could produce remissions in children with acute leukaemia. Thereafter, folate-dependant enzymes became a major target for chemotherapy as they play an essential role in the synthesis of DNA precursors(Chu et al., 1996, Huennekens, 1994). Methotrexate is the most commonly used anti-folate for the treatment of leukaemia, lymphoma, chorio-carcinoma, head and neck cancer and osteogenic sarcoma. It is additionally used in the treatment of numerous autoimmune diseases, including rheumatoid arthritis, psoriasis and the prevention of graft-host diseases after transplantations (Gorlick et al., 1996). Methotrexate and its polyglutamates bind tightly to dihydrofolate reductase, inhibiting the enzyme.

This results in the depletion of N^5N^{10} methylene tetrahydrofolate and N^{10} formyl tetrahydrofolate, essential cofactors for the biosynthesis of thymidylate and purine respectively (Chu et al., 1996). The depletion of these cofactors results in the inhibition of DNA replication and ultimately cell death. Furthermore, methotrexate polyglutamates also inhibit the enzymes involved in the *de novo* synthesis of purines, glycinamide ribonucleotide (GAR) transformylase and aminoimidazole carbozamide (AICAR) transformylase. Additionally, methotrexate competes with reduced folates for transport and polyglutamates (Chu et al., 1996). However, the genetic origins of inter-individual pharmacokinetic and pharmacodynamic variability of methotrexate remain poorly understood, with conflicting results on candidate pharmacogenetic predictors for methotrexate (de Jonge et al., 2005, Laverdiere et al., 2002). *SLCO1B1* has been shown to transport methotrexate *in vitro* (Abe et al., 2001, Tirona et al., 2001). Previous studies have confirmed the functional consequences of nonsynonymous polymorphisms in *SLCO1B1* (Kameyama et al., 2005, Tirona et al., 2001). Trevino et al. (2009) concluded that for RS 4149056C, as well as other less studied SNPs, a lower methotrexate clearance was observed. This is consistent with numerous preceding studies which demonstrated greater plasma exposure to several *SLCO1B1* substrates in those carrying the RS4149056C polymorphism (Ho et al., 2007, Niemi, 2007, Pasanen et al., 2007).

1.10.3. HMG-CoA Reductase inhibitors (Statins)

Statins are the most commonly prescribed lipid-modifying therapy for hyperlipidaemia (Downton and Clark, 2003). Numerous largescale studies have proven the advantageous effects of statins in primary and secondary prevention of CVD (Law et al., 2003, Sacks et al., 1996, Shepherd et al., 1995, Strandberg et al.,

2001). Statins are reversible competitive inhibitors of the HMG-CoA reductase enzyme. Inhibition of this enzyme results in a reduction of cholesterol synthesis in hepatocytes, which leads to a reduction in intracellular cholesterol concentrations and therefore an increase in LDL-receptor expression on the hepatocyte cell surface (Law et al., 2003). The result is an increase in extraction of LDL cholesterol from the blood and therefore a decrease in the total cholesterol concentration in the circulatory system (Law et al., 2003). Additionally, statins also moderately reduce triglyceride concentrations and increase HDL cholesterol concentrations. However, the biological effects, other than LDL reduction, may vary between statins (Bonetti et al., 2004, Chong et al., 2001, Rosenson and Tangney, 1998). Statins have been shown to slow the progression, or even promote regression, of coronary artherosclerosis and avoid plague that would elicit intramural haemorrhage and intraluminal thrombosis (Kreisberg and Oberman, 2002, Liao et al., 2002). Included in the statin class are Fluvastatin, Pravastatin, Simvastatin, Rosuvastatin and Atorvastatin.

1.11. OCTs and MATEs in drug-drug interactions

OCTs and MATEs play an important role in the uptake, distribution, and elimination of commonly used clinical drugs. Since more than 30% of clinically used drugs are organic cations, drug-drug interaction (DDI) by inhibition of OCT and/or MATE transporters may be clinically relevant. DDIs involving the inhibition of metabolism and/or excretion prolong the plasma elimination half-lives, leading to the accumulation of victim drugs in the body, and consequently potentiate pharmacological/adverse effects (Ito et al., 2012).

Apical efflux by the MATE family is considered one of the sites of DDI in addition to OCTs at the basolateral membrane (Tsuda et al., 2009). The antihistamine cimetidine is known to cause DDIs with OCs in the kidney, and a previous study showed that the co-administration of cimetidine with fexofenadine (FEX), for example, decreases the renal clearance of the drug. Previously hOCT2 was implicated in DDIs which involved the antihistamine cimetidine. In a study by Matsushima *et al*. (2009) it was suggested that the DDI with cimetidine and FEX was mainly caused by the inhibition of hMATE1mediated efflux of FEX rather than the inhibition of its renal uptake process (Matsushima et al., 2009). Moreover, a recent study by Ito *et al.* (2012) supported this observation and showed that it is in fact the competitive inhibition of the luminal efflux by hMATE1, and not the basolateral uptake by hOCT2, which is the likely mechanism underlying the pharmacokinetic DDIs caused by cimetidine in the kidney (Ito et al., 2012). Inhibitors that preferentially interact with and impair the function of MATEs, may not only result in decreased clearance but may also lead to nephrotoxicity.

Minematsu *et al*. (2011) investigated the inhibitory effect of 8 tyrosine kinase inhibitors on metformin transport activity by human hOCT1, hOCT2, hOCT3, hMATE1 and hMATE2K (Minematsu et al., 2010). They found that imatinib, nilotinib, gefitinib, and erlotinib exerted selectively potent inhibitory effects on hMATE1, hOCT3, hMATE2K, and hOCT1, respectively. Furthermore, they found that compared to the reference hOCT1, the M420del variant was more sensitive to drug inhibition erlotinib.

1.12. Genomic Diversity and Personalized Medicine: The African Perspective

Although controversial, the concepts of race, ethnicity, and ancestry, have for a long time been recognized as having a strong influence on pharmacogenomic discovery, and our understanding of population differences in drug efficacy and toxicity (Urban, 2010).

Most dosing regimens are recommended on the basis of clinical trials that have been conducted in Caucasian or Asian populations which may not be appropriate for African populations (Masimirembwa and Hasler, 2013). Pharmacogenomic applications hold the promise of using genome-based technologies to improve health by the prevention or effective treatment of disease. The current belief is that even developing nations, such as those in sub-Saharan Africa, can benefit from pharmacogenomics in order to inform public health policies, designing and interpreting clinical trials, and possibly to help guide clinicians to prospectively evaluate those patients with the greatest probability of expressing a variant genotype (Suarez-Kurtz, 2008b, Daar and Singer, 2005a).

The South African health system, both private and public, faces a high burden of communicable and non-communicable diseases, high maternal and child mortality, as well as injury and violent related deaths (Coovadia et al., 2009, Mayosi et al., 2012). This high burden of infectious and chronic diseases results in a health system that is continuously under-resourced. Although South Africa's per capita health expenditure is the highest of any middle-income country in the world its health outcomes are often worse than that of lower-income countries (Coovadia et al., 2009, Warnich et al., 2011). The current challenge in pharmacogenomics in

sub-Saharan Africa, and for that matter South Africa, is to ascertain the extent of the genomic diversity in our understudied populations, to understand genotype-environment interactions, and to translate this knowledge into clinical applications that can be utilized in public health care (Hardy et al., 2008).

1.13. Usage of Manuscript Data

Data from the following manuscripts was used in **Chapter 2**: **Pearce et al** (2016) and **Du Plessis et al** (2015). Data from **Hoosain et al** (2016) was used in **Chapter 3.**

1.14. Summary and Main Objectives of the Project

The past decade has seen remarkable progress in the field of membrane transporters, not only in terms of functional characterization and substrate specificity but also in elucidating the important role that transporters play in diseases such as cancer and in the disposition and efficacy of drugs in clinical use. To date 48 members of the ABC and over 325 members of the SLC families of membrane transporters have been identified. These transporters are ubiquitously expressed and play a critical role in maintaining cellular and organismal homeostasis by importing nutrients essential for cellular metabolism and eliminating metabolic by-products and toxic xenobiotics. Whereas most membrane transporters are oligo-specific (specialized for the translocation of specific metabolic or nutritional compounds), poly-specific transporters accept compounds with different sizes and molecular structures. These poly-specific transporters exhibit large variations in affinity and turnover for different compounds and may have specific physiological roles. Given the fact that 40% of clinically used drugs exist as organic cations at physiological pH, the poly-specific OCT and MATE families of SLCs have attracted a significant amount of

interest. The genes encoding these transporters are also being investigated as potential risk loci for cancer, and SNPs in these genes have been associated with imatinib treatment failure in CML patients. Moreover, genetic variations in these transporters are increasingly being recognized as a possible mechanism that can explain the inter-individual variability in drug efficacy and toxicity. However, the majority of these studies were conducted in Caucasian and Asian populations and were based on genetic variants that are specific to these populations. The findings of these studies are often extrapolated for use and interpretation in other populations. This is in spite of the fact that the population frequency of variant alleles can differ markedly between populations. In addition, ethnic-specific variants exist in non-Caucasian and non-Asian populations which may be more predictive of treatment outcome or disease progression for a specific ethnic group. However, the allelic distribution and role of genetic variants of OCT, OAT and MATE transporters in drug efficacy and toxicity and disease progression in indigenous South African populations have not received the necessary priority.

Inter-individual variability in drug response is a significant clinical problem which has attracted a fair amount of research interest. It is estimated that genetic factors can account for approximately 15-30% of inter-individual variability in drug response and efficacy. Initially the focus has been on drug metabolizing enzymes, however membrane transporter belonging to the SLC family are increasingly being recognized as a possible mechanism explaining this variability in drug response and efficacy. Given that 40% of therapeutic drugs exist as OCs at physiological pH, the OCTs, OATs and MATEs of the SLC family is receiving a greater amount of attention. Reduced-function variants have been identified, primarily in American and European Caucasians, which affect the

pharmacokinetics of OCT and MATE substrate drugs such as metformin for example. Although African populations are considered to harbour more genomic diversity than Caucasian populations, this diversity is however understudied. Thus, little or no information is available on the extent of genetic variation in *OCT, OAT* and *MATE* genes within African populations. Although several populations from West, East, and Central Africa are included in the 1000 Genomes and Hap-Map projects, these groups cannot represent the genomic diversity of the entire continent. Therefore, in order to bridge the gap that exist with regards to information on the pharmacogenomic relevant OCT and MATE genes, especially in indigenous southern African populations, this study prioritized the genotyping of 78 SNPs in four genes, known to affect drug disposition and efficacy, in the Cape Admixed population of South Africa.

The main aim of this study was to evaluate the distribution of genetic variants in solute carrier transporters amongst the Cape Admixed population and this aim was achieved through the following objectives;

(1) Investigating the genotypic and allelic distributions of thirty-eight SNPs, and to infer the haplotype structure of the *SLC22A1*-3 genes.
(2) Determining the baseline genotypic and allelic frequency distributions of 20 known SLCO1B1 coding SNPs.
(3) To determine whether the SLC genes of the Cape Admixed population harbours any novel SNPs, using direct sequencing of the promoter regions of these genes in 96 healthy individuals. Secondly to determine the promoter haplotype structure of the SLC genes based on the genetic information acquired by sequencing.

(4) Investigate the effect promoter haplotypes have on gene expression of *SLC22A2*.

CHAPTER 2

Genetic Polymorphisms and Haplotype Structure of *SLC22A* Genes in the Cape Admixed Population

2.1. Abstract

Human organic cation transporters are primarily expressed in hepatocytes and mediates the electrogenic transport of various endogenous and exogenous compounds, including clinically important drugs. Genetic polymorphisms in these genes are increasingly being recognized as a possible mechanism explaining the variable response to clinical drugs, which are substrates for this transporter. The genotypic and allelic distributions of nonsynonymous solute carrier transporter single nucleotide polymorphisms were determined in 130 healthy Cape Admixed participants from South Africa, using a SNaPshot™ multiplex assay. In addition, haplotype structure for each gene (*SLC22A1, SLC22A2, SLC22A3*) was inferred from the genotypic data. The minor allele frequencies for *SLC22A1* variants S14F (rs34447885), P341L (rs2282143), V519F (rs78899680), and the intronic variant rs622342 were 1.0%, 0.5%, 1.5%, and 18.0%, respectively. The minor allele frequencies for the *SLC22A2* variants S270A (rs316019) and R176H (rs57371881) were 7.1% and 4.5%, respectively. None of the participants carried the variant allele at any of the elected loci in *SLC22A3*. This study reports important genetic data that could be useful for future pharmacogenomic studies of drug transporters in the indigenous Sub-Saharan African populations.

CHAPTER 2

2.2. Introduction

Membrane transporters play an important role in the metabolism of clinical drugs and endogenous compounds. Single nucleotide polymorphisms (SNPs) in ATP-binding cassette (*ABC*) and solute carrier transporter (*SLC*) genes have been increasingly recognized as a possible mechanism accounting for altered transport activity, which may have profound clinical implications (Leabman et al., 2003a). In general, genetic factors are estimated to account for 15-20% of inter-individual variations in drug disposition and responses (Choi and Song, 2008a, Evans and Relling, 1999, Eichelbaum et al., 2006). However, for certain drugs genetic factors can account for up to 95% of inter-individual variability in drug disposition and effect (Eichelbaum et al., 2006, Evans and Relling, 2004).

Poly-specific organic cation transporters (OCTs) are involved in the sodium-independent electrogenic transport of small organic cations (OCs) with different molecular structures (Koepsell et al., 2007b). These organic cations include clinically important drugs (metformin, cimetidine, procainamide), endogenous compounds (dopamine, norepinephrine, and toxic substrates (tetraethylammonium, haloperidol-derived pyridinium metabolite, 1methyl4phenylpyridinium) (Gorboulev et al., 1997a). Based on their substrate properties and tissue distributions, human OCT13 are thought to play important roles in the biliary and renal excretion of their substrates and the distribution of organic cationic drugs in the liver, kidney, heart, and brain (Jonker and Schinkel, 2004).

The uptake transporter hOCT1 is encoded by the *SLC22A1* gene which is located on chromosome 6q26, and consists of 11 exons spanning approximately 37kb (Gorboulev et al., 1997a, Koehler et al., 1997a, Koepsell et al., 2007b). hOCT1 is

primarily expressed in the sinusoidal or basolateral membrane of hepatocytes and is thought to play an important role in the hepatic uptake, distribution and excretion of clinically important drugs (Gorboulev et al., 1997a, Zhang et al., 1997).

The role of hOCT1 in the clinical pharmacology of clinical therapeutics such as the antidiabetic drug metformin, the antineoplastic imatinib, the anti-HIV drug lamivudine, and the serotonin receptor type antagonists tropisetron and ondansetron has been extensively researched (Shu et al., 2007, Shu et al., 2008, Tzvetkov et al., 2009). Moreover, a number of *SLC22A1* variants have been associated with functional changes in protein activity, as well as drug disposition, response, and toxicity. For example, Bazeos *et al*. (2010) found that *SLC22A1* transcript levels and SNPs can be predictive factors for response to imatinib in chronic myeloid leukemia (CML) (Bazeos et al., 2010). Previous studies have shown that hOCT1 is highly polymorphic in ethnically diverse populations (Sakata et al., 2004, Shu et al., 2003, Kang et al., 2007a).

The hOCT2 gene SLC22A2, consisting of 11 exons, was first cloned in 1997 and is located on chromosome 6q26, adjacent to SLC22A1 which encodes for hOCT1 (Gorboulev et al., 1997a, Koehler et al., 1997a, Koepsell et al., 2007b). The hOCT1 paralogue hOCT2 consists of 555 amino acid residues and has been detected in the kidney, placenta, spleen, intestine, and neuron (Busch et al., 1998, Gorboulev et al., 1997a, Koepsell et al., 2007b)

Examples of clinically important drugs transported by hOCT2 include the antidiabetic drugs metformin and phenformin, the antineoplastic drugs cisplatin and oxaliplatin, the anti-HIV drugs lamivudine and zalcitabine, and the histamine receptor antagonist cimetidine (Barendt and Wright, 2002, Busch et al., 1998,

Ciarimboli et al., 2005b, Dresser et al., 2002, Kimura et al., 2005b, Jung et al., 2008). In addition, hOCT2 is also responsible for the transport of endogenous compounds such as monoamine neurotransmitter 5-hydroxytryptamine (5-HT), agmatine, choline, dopamine, epinephrine, histamine, and norepinephrine, as well as compounds such as creatinine (Amphoux et al., 2006, Busch et al., 1998). Furthermore, this transporter is critical in the detoxification and elimination of xenobiotics from the systemic circulation and is also involved in the transport of toxic substances such as tetraethylammonium bromide (TEA), HPP+, and 1methyl4phenylpyridinium (MPP+) (Burckhardt and Wolff, 2000, Gorboulev et al., 1997a, Okuda et al., 1999, Otsuka et al., 2005, Zhang et al., 1997, Zhang et al., 1998)

The human organic cation transporter 3 (hOCT3), also known as extra-neuronal monoamine transporter (EMT), has a broad distribution and is found in various tissues, including the liver, heart, placenta, skeletal muscle, kidney, and brain (Gründemann et al., 1998, Wu et al., 2000). Moreover, hOCT3 is a poly-specific transporter that is involved in the cellular uptake and elimination of small OCs with different molecular structures. These OC substrates include endogenous bio-amines, clinically important drugs and xenobiotics. Examples of substrates transported by hOCT3 include the antidiabetic metformin, the biogenic amines histamine, dopamine, and epinephrine, and the xenobiotics tetraethylammonium bromide (TEA) and the neurotoxin 1methyl4pyridinium (MPP^+) (Martel 2003).

The gene encoding for hOCT3, SLC22A3, encodes a protein consisting of 556 amino acid residues and is located on chromosome 6 where it is clustered together

with SLC22A1 and SLC22A2 the genes coding for hOCT3's paralogues hOCT1 and hOCT2, respectively (Koehler et al., 1997a, Verhaagh et al., 1999).

These aforementioned reduced-function genetic variants were however primarily found in studies with European participants and have not been consistently replicated for other ethnic groups (Chen et al., 2010b, Leabman et al., 2003a). Recent reports using genome-wide polymorphisms suggested that: (i) genetic variation seen outside of Africa is generally a subset of the total genetic variation that exists within Africa, (ii) genetic diversity decreases with increased geographic distance from Africa, and (iii) linkage disequilibrium (LD) patterns increase proportionally to the distance from Africa (Jakobsson et al., 2008, Li et al., 2008, Tishkoff et al., 2009). Moreover, Rosenberg *et al* (2002) found that there is greater genetic diversity among African populations compared to Caucasian or Asian populations (Rosenberg et al., 2002). However, despite Africa harboring a significant proportion of human genomic diversity, this genomic diversity is unfortunately relatively understudied (Hardy et al., 2008, Tishkoff et al., 2009).

South Africa is home to a large number of indigenous and immigrant population groups (Hardy et al., 2008, Benjeddou, 2010). Amongst these are the indigenous Khoisan, Xhosa, Zulu, Venda, and Sotho Pedi groups, the Afrikaners and the Cape Coloured, the latter being a uniquely admixed population of immigrant Europeans, Asians and the indigenous populations(Hardy et al., 2008). Admixed groups, such as Latinos, African Americans, or Cape Coloureds from South Africa, share varying proportions of different ancestral populations and their genetic complexity can potentially complicate biomedical research studies (Via et al., 2009a). This genomic diversity could provide a wealth of information and knowledge, which could

eventually be applied to aid our understanding of the impact of genetic variation on complex diseases such as cancer, diabetes mellitus, hypertension and the inter-individual variability in response of patients to drugs used in the treatment of these diseases. Although limited, studies that have been conducted in South Africa suggest that South African populations have unique genetic profiles which include novel and rare variants, with allele frequencies differing from each other and other African populations (Warnich et al., 2011).

Previous studies have shown that South African populations exhibit unique allele frequencies and novel genetic variations in pharmacogenomically relevant genes (Ikediobi et al., 2011). However, these studies have primarily focused on variants in drug metabolizing enzyme genes. Information on variants in drug transporter genes for South African populations is however limited or nonexistent. Therefore, the aim of this study was to investigate the genotypic and allelic distributions of thirty-eight SNP(s), and to infer the haplotype structure of the *SLC22A1-3* genes in the CA population.

2.3. Materials and Methods

2.3.1. Subjects

Samples were obtained from the participants with informed consent. This study was approved by the Senate Research Ethics Committee of the University of the Western Cape, South Africa. Biological samples were collected in the form of buccal swabs from 130 unrelated healthy volunteers from the CA population. Ethnicity of volunteers was determined by self-report.

2.3.2. DNA extraction

CHAPTER 2

Genomic DNA was isolated from buccal swab samples using a standard salt lysis protocol and stored frozen at 20°C until the time of genotyping (Leat et al., 2004).

2.3.3. SNP selection

A total of 20 OCT1 gene SNPs (19 nonsynonymous and 1 intronic), 20 OCT2 gene nonsynonymous SNPs, and 18 OCT3 gene coding SNPs were selected for this study. SNPs were selected from the literature and the Ensembl database (http://www.ensebl.org) (Flicek et al., 2012). OCT1 variants A306T, A413V, M420V, C436F, I421F, V501E, V519F, and I542V, OCT2 variants M1V; R176H; C282G; L351W; R207H; T357M; M393T; R404C; G439E; R463K; R487Q R487W; V502E/G; and V502M, and OCT3 variants N162I; A169T; R212H; M248V; G269E; R293C; R310C; S337F; R348W; I381T; V388M; R403H; R407H; I431K; and R490Q were included in this study based on predicted effect on function, using the SIFT (Sorting Intolerant From Tolerant) program (Flanagan et al., 2010, Kumar et al., 2009, Ng and Henikoff, 2003). To our knowledge no population data exist in the public domain for these variants.

2.3.4. Primer design

Multiplex PCR primers, listed in **Tables 2.3.12.3.3**, were designed to have an annealing temperature between 55°C and 60°C using Primer3 software (www.genome.wi.mit.edu/cgibin/primer/primer3). To test for possible nonspecific amplification, primers were aligned with the NCBI sequence databases using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast/blastcgi). Two SNaPshot® Multiplex systems were specifically designed, for each gene, successfully optimized and used for genotyping. The single base extension primer sets for multiplex 1 and 2 are listed in **Tables 2.3.4** (OCT1), **Table 2.3.5** (OCT2) and **Table 2.3.6** (OCT3).

Table 2.3.1 Multiplex PCR primers for the generation of *SLC22A1* amplicons used in SNaPshot™ genotyping.

Location	Forward primer (5´to 3´)	Reverse Primer (5′to 3′)	Amplified region (NC_000006.12)	Amplicon length (bp)
Exon 1	TGCTGAGCCATCATGCCCACCGTG	GGACACAGCCAGACACCCACG	160121924 - 160122483	560
Exon 2	CTCTTGCCGTGGTATGACTGGCAG	CAGAGGGGCTTACCTGGACTGG	160130080 -160130240	161
Exon 3	CCTCCATGTCTCCTTCTCTCTGAAG	CTGGCCTCATCCCCATGATAATTAC	160132207 -160132411	205
Exon 4	CCCGCATAACGTCCACACCTCCTG	GTAGGCAGGAGGAAGGGCCTCAC	160133927 -160134148	222
Exon 5 & 6	GATAGTGATGAGTGGTGTTCGCAG	GCGAGCGTGCTGATTCTGCCT	160136196 -160136698	503
Exon 7	GACTTGAAACCTCCTCTTGGCTCAG	TTCCCCACACTTCGATTGCCTGGGA	160139628 -160139923	296
Exon 8	GAAGCCCCCATCCACCACCCACACC	GGCTACCCCTGTTCCATGCACTCAC	160143495-160143674	180
Exon 9	ATTGCATGGGCAACGGATGGCT	CCATGCTGAGCCCACTGCCGAGCTG	160154557-160154972	416
Intron 9	GAGTAGGAGGGGTTAATAGAGAGAG	GTAGCTGAGACTACATGCATGCACCAC	160151769-160152004	236
Exon 10	TTCCTCTCTTTGGCTGGCTGTGA	ACTCCAGCAAACCTTGCTCTCTGT	160155888-160156508	621
Exon 11	TGCCCTTTTCTTCTTTGCTGTTTGC	AGCACCAACAGCTTTCCCTAGATCG	160158364-160158823	460

Table 2.3.2 Multiplex PCR primers for the generation of *SLC22A2* amplicons used in SNaPshot™ genotyping.

Location	Forward primer (5´to 3´)	Reverse Primer (5′to 3′)	Amplified region (NC_000006.12)	Amplicon length (bp)
Exon 1	TTTGGGAAGTGCAGAAGGAC	CCATTTGCTTCTCCATCTGAG	160258972 - 160258303	670
Exon 2	GGAACACTTCTCCCCTGCT	CACCACAGGTGATTCAACCTAC	160256741 - 160256592	150
Exon 3	GTGAATGGGGCTTATCATGC	TCTATTTTGGCAGCGAGGTT	160250933 - 160250491	443
Exon 4	CAGGCCTTTCATCCCATCTA	GGGTCTGGAGAGTGAAAGCA	160249613 - 160249098	516
Exon 5	GGATGGGGTAAGGAGGATTC	TTTCTCCATCCCCTGATTTG	160247353 - 160247143	211
Exon 6	TGACCCAGGGACACTAGCAT	TACCGGGATGAGGTCATGTT	160245603 - 160245344	260
Exon 7	CACAGCCAGCCACTGAAGTA	GCTGGCCATATGAATTTGCT	160243961 - 160243408	554
Exon 8	ATTCTGGGATGGGGAATTTG	TCCTTTGTCTGCACTTGTGG	160242504 - 160242212	293
Exon 9	AGGGGTGGATGGGAGATAAC	ACATCCAGGAAGAACGCAAG	160241655 - 160241313	343
Exon 10	TTCAATGGAGTTTGGAACTGG	TGAATTTATCTCAGTGTATGGTGTGA	160224991 - 160224588	404
Exon 11	AATTTCTTTCTCCCCTCTCCA	TTTTAAAATCCACAAATGTTAAGACA	160217538 - 160216700	839

Table 2.3.3 Multiplex PCR primers for the generation of *SLC22A3* amplicons used in SNaPshot™ genotyping.

Location	Forward primer (5´to 3´)	Reverse Primer (5′to 3′)	Amplified region (NC_000006.12)	Amplicon length (bp)
Exon 2	TGCATTCTGGCATGTCTCCATGTGT	ACCGGGAACAGCCTCAGACCT	160397935 - 160398311	377
Exon 3	GTTTAAGGTGAGCTCTTTTCCTGT	TTGGCTCCCAAAGTAAGGTGG	160407004 - 160407404	401
Exon 4	CTGCAAGTGTGGAAGCCTCCGT	GCTGGGCAGCGTGATGGCTA	160408607 - 160408898	292
Exon 5	TGCAGGAATAATCTGTATTTCAGGG	ACTGAAAATGATTTCCCAGATGTT	160410569 - 160411034	466
Exon 6 & 7	TGAAAGCCCCTAGTCACTTCAG	TGGAGTGACATCACGAAAGACT	160436664 - 160437340	677
Exon 8	CTTCAGACTGGAGGCCACTAAGCA	ACGCTGGTCTACAGAGTTACTTAG	160442659 - 160442921	263
Exon 9	GGATAACACCCTCCACCCAC	ACTGAATTGGCTCTCAAAACTG	160443405 - 160443934	530
Exon 10	TGTTTCCCTGTGATGCAGGA	TGCTTCTCTCTTCACAACCACAT	160447401 - 160448051	651
Exon 11	TGATCCTGGAGACAGATATTGTTGT	GTCAGAGACCACAGGGAACA	160450844 - 160451347	504

2.3.5. Multiplex PCR

The PCR reactions were performed in a 20 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qiagen multiplex PCR master mix (Qiagen, Courtaboeuf, France) and 0.2 μM of each primer. Cycling consisted of an initial 15-minute activation step for HotStar Taq polymerase at 95°C, followed by a total of 35 cycles using the following conditions: 94°C denaturation for 30 seconds, primer annealing at 60°C for 90 seconds, and primer extension at 72°C for 30 seconds, and 15 minutes of final extension at 72°C and a 4°C holding step. PCR products were purified to remove excess primers and unincorporated dNTPs using an Exo/SAP protocol. The entire 20 μl of PCR products were incubated with 0.5 μl of Exo1 and 1 μl of FastAP for 30 minutes at 37°C followed by 15 minutes at 80°C for enzyme inactivation. PCR quality and yield were checked using Nano-Drop.

Table 2.3.4 *SLC22A1* multiplex 1 single base extension primers.

NCBI (dbSNP)	Amino Acid Change	Nucleotide Change	Single Base Extension Primers (5′ to 3′)	Position Accession number (NC_000006.12)	Size bp	Poly-GACT tail
Multiplex 1						
rs34447885	S14F	C/T	TGACTATTCTGGAGCAGGTTGGGGAGT	160121976	40	13
rs34104736	S189L	C/T	GAACTGTGCTGGTCAACGCGGTGT	160132282	45	21
rs36103319	G220V	G/T	GGTCAGCAAGGGCAACTGGATGGCTG	160132375	50	24
rs4646277	P283L	C/T	GATAACAGCCACCGGGGGACACC	160136228	55	32
rs34130495	G401S	G/A	AGCCCTCATCACCATTGACCGCGTG	160139792	60	35
rs72552763	M420V	A/G	AACTTACCAGGTGAGATAAAAATCA	160139849	65	40
rs35956182	M440I	G/A	CATAATCATGTGTGTTGGCCGAAT	160143584	70	46
rs34059508	G465R	G/A	CCACAGGGAGGAACACACCATCACTC	160154805	75	49
rs78899680	V519P	G/T	CTACTTCTTCCAGAGACCAAGGGG	160156031	80	56
rs137928512	I542V	A/G	CAGAGGTTTGGACCTTAAGGTAAA	160158541	85	61
Multiplex 2						
rs622342	Intron	A/C	ATTTCTTCAAATTTGATGAAAACTTC	160151834	14	40
rs12208357	R61C	C/T	TCCTGGGGTGGCTGAGCTGAGCCAG	160122116	20	45
rs4646278	R287G	C/G	CAGTGTTTCTTTTTTGTGATAACAGCCACC	160136239	20	50

rs55918055	C88S	T/A	TCCAGTCCACTTCATAGCGCCTGC	160122197	31	55
COSM164365	A306T	G/A	AGGAGGCAACTTCCCATTCTTTTGAG	160136296	34	60
rs2282143	P341L	C/T	CTTCATTTGCAGACCTGTTCCGCACGC	160136611	38	65
rs144322387	A413V	C/T	CCCCATGGCCATGTCAAATTTGTTGG	160139829	44	70
rs151333280	I421F	A/T	CCAACTTACCAGGTGAGATAAAAA	160143571	51	75
rs139512541	C436F	G/T	GCACTGGTTAAACATCATAATCATGT	160143571	54	80
rs143175763	V501E	T/A	CACTCCCGCGGCAAGCAGGCCCAAC	160155979	60	85

Table 2.3.5 *SLC22A2* gene multiplex 1 single base extension primers for SNaPshot™ genotyping assay of selected SNPs.

NCBI (dbSNP)	Amino Acid Change	Nucleotide sequence (5′ → 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	Poly-GACT tail
Multiplex 1					
rs57371881	R176H	ATGAGGACTGTAGTTAGGAGGCAGAGCTTA	rs57371881	30	0
rs201919874	T199I	CAGCTGGAGTTCTCATGGCCATTTCCCCAA	160250625	35	5
rs8177508	M165V	TCAGTGAATGTAGGATTCTTTATTGGCTCT	160256639	40	10
rs144729356	C282G	AGATCAGCCACCTGGGAGACTCAGGTATGC	160247297	45	15
rs45599131	L351W	GTGAAAAATATTCCTTACCAGTTGTACATC	160245451	50	20
rs8177517	K432Q	TCTTTTCCCTCTTAGATCTACAATGGCTA	160242388	55	25
rs145450955	T201M	CCTTGGATTAAGCGAAAAATTAACATCCAC	160250619	60	30
rs316019	S270A	CCTCACTGGAGGTGGTTGCAGTTCACAGTT	160249250	65	35
rs8177516	R400C	ATGCAGCCCAAGGGTAACGGCGTCCGATGC	160243653	70	40
rs141582772	M1V	GCAGCCTCGGGCCCTCCTGCCTGCAGGATC	160258757	75	45

Multiplex2

rs141205337	M393T	CGTCCGATGCGGTCGATGGTGAGGATGATC	160243673	30	0
rs141405449	G439E	ATCTCATAGGCCATTGTGATCCCCATTCTT	160242366	35	5
rs3907239	R463K	CCCTCGTCATTCTAAGGAAAATGCACTCAC	160242294	40	10
rs184227446	T357M	ATGATGAGGCCCTGGTAGAGCACAGAGCTC	160243781	45	15
rs140829992	R487W	GGTGGCATCATCACGCCATTCCTGGTCTAC	160241516	50	20
rs17853948	V502E/G	AGCACCAGACCTCCAGCAACCAAGCCAAGC	160224801	55	35
rs137885730	R404C	CAACCATATTTGATGCAGCCCAAGGGTAAC	160243641	60	30
rs140033522	V502M	ATGTGATATTCATCTGTTTGGCTTTCAGGC	160224802	65	25
rs151282335	R487Q	AGCGGGAGCTCAAGCCAGATGTTAGTGAGC	160241515	70	40
rs199783132	R207H	GCTGCTTTGCTGACCAGTCCTTGGATTAAGC	160250602	75	45

Table 2.3.6 SLC*22A3* gene single base extension primers for SNaPshot™ genotyping assay of selected SNPs.

NCBI (dbSNP)	Amino Acid Change	Nucleotide change	Nucleotide sequence (5′ → 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	Poly-GACT tail
Multiplex 1						
rs183669984	R310C	C>T	AAAGGAGATAAAGCATTACAGATCCTGAGA	160410799	30	0
rs137958808	M370I	G>T	AAGCGCAGTGGTGTATCAAGGACTTGTCAT	160437033	35	5
rs199688797	R212H	G>A	CACCAAACTTCCCTGTGTTTGTGATCTTCC	160407142	40	10
rs150004342	A169T	G>A	ACCTGTCTGCTGCATAGCCTAAGGTGAATG	160398054	45	15
rs142228053	R293C	C>T	TTCTTTGCCAGGGTGGTCCCTGAGTCTCCC	160410748	50	20
rs149424049	I431K	T>A	AATGTAGCCACTGTGGTCCTCAACCATGCT	160442764	55	25
rs147863404	G269E	G>A	AAGCTGGGCAGCGTGATGGCTAACTGGATT	160408870	60	30
rs8187725	T400I	C>T	AAGGGGAGGCGTCGTCCAAGGCGCTCAATG	160437122	65	35
rs149101094	M248V	A>G	CAAAGGAGGATTGTGGGAATCGTGATTCAA	160408806	70	40
rs141104413	S337F	C>T	CTGTTACAGATGAGGAAGTTAGTAATCCAT	160436814	75	45

Multiplex 2

rs139266499	N162I	A>T	GGATGCTGGACCTCACCCAAGCCATCCTGA	161081878	35	5
rs145328121	R348W	A>T	TTTTTAGATCTGGTGAGAACTCCCCAAATG	160436846	45	15
rs187750009	I381T	T>C	GCCTGGGAATTATAGGGGGCAACCTCTATA	160437065	50	20
rs189883656	V388M	G>A	AGATCAAGAGAGCTCCTGGCAGTTCCACCA	160437085	55	25
rs200478210	R403H	G>A	GAGCTCTCTTGATCTTACTAACCATTGAGC	160437131	60	30
rs145082363	R407H	G>A	GCCACTATATTGCTTGCCGCAAAGGGGAGG	160437143	65	35
rs12212246	A439V	C>T	CAGGAATAGCATGGTTGAGGACCACAGTGG	160442788	70	40
rs144856002	R490Q	G>A	AGAGGTAGTTCTAGCCACACGGCTGCTAGC	160443701	75	45

2.3.6. Multiplex mini-sequencing reactions

Multiplex mini-sequencing was performed in a 10 μl reaction volume using 3 μl of a 1/10 dilution of purified PCR products, 0.10.2 μM of primers, and 5 μl of SNaPshot® (ThermoFisher Scientific, California, USA) ready reaction mix. Sequence cycling consisted of 25 cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds, and primer extension at 60°C for 30 seconds. Post-extension treatment was done by adding 1 U of FastAP to the 10 µl reaction volume and incubation at 37°C for 30 minutes followed by 15 minutes at 80°C to deactivate the enzyme.

2.3.7. Electrophoresis of the mini-sequencing products

The purified mini-sequencing products (1 μl) were mixed with 8.7 μl of HiDi™ formamide and 0.3 μl of GeneScan120 Liz size standard (Also observed in the promoter region within the Xhosa population (Jacobs et al., 2015)) and denatured at 95°C for 5 minutes. The fluorescently labelled fragments were separated on 36 cm long capillaries in POP4 polymer on an ABI Prism 3500 Genetic Analyzer (Also observed in the promoter region within the Xhosa population (Jacobs et al., 2015)). Data analyses were performed using GeneMapper® IDX Software Version 1.2.

2.3.8. Statistical Analysis

Genotype and allele frequencies as well as the deviation from the Hardy-Weinberg Equilibrium were calculated using GenAlEx 6.5 software (Peakall and Smouse, 2012, Wigginton et al., 2005). Allele and genotype frequencies are given with binomial proportion 95% confidence intervals (CI) calculated according to the method of Wilson. The SHEsis analysis platform was used to infer the haplotype

frequencies (Yong and Lin, 2005, Li et al., 2009). Statistical significance was defined as $p<0.05$.

2.4. Results

The population studied consisted of 130 Cape Admixed individuals between the ages of 18 and 61 years. There were 46 (35%) female and 84 (65%) male participants. The mean age of female participants was 25.3±9.0 years, whereas male participants had a mean age of 24.8±7.7 years.

OCT1

The genotype and allele frequencies of the 20 OCT1 gene SNPs investigated in the 130 Cape Admixed subjects are summarized in Table 2.4.1. The allelic frequency of each SNP was in HWE ($p>0.05$), except for rs622342. Twelve of the nineteen investigated nonsynonymous SNPs were monomorphic in the CA population. None of the participants were homozygous for the variant allele for S14F (rs34447885), P341L (rs2282143), and V519F (rs78899680). The S14F variant genotype frequencies for homozygote wildtype (CC), heterozygote (CT) and homozygote (TT) were 98%, 2% and 0.0%, respectively. The MAF observed for S14F was 1%. The P341L variant genotype frequencies, on the other hand, for homozygote wildtype (CC), heterozygote (CT) and homozygote (TT) were 96%, 1% and 0.0%, respectively. The S189L and G220V variants displayed the same genotype frequencies for their respective genotypes; 98%, 2% and 0.0%, respectively. Additionally, the M440I and G465R variants displayed the same genotype frequencies for homozygote wildtype (GG), heterozygote (GA) and homozygote (AA); 99%, 1% and 0.0%, respectively for each. Whereas the genotype frequencies

for variant V519F were 97% (homozygote wildtype (GG)) and 3% (heterozygote (GT)). Variants R61C (rs12208357), C88R (rs55918055), P283L (rs4646277) and G401S (rs34130495) were not observed in the Cape Admixed nor the Native American/Hispanic Admixed populations. The S14F variant was observed in the Cape Admixed and other Sub-Saharan populations, but not in all other populations. The S189L and G220V variants were only observed in the Cape Admixed population at a frequency of 1%.

The inferred haplotypes for investigated population are listed in Table 2.4.3. The most frequently observed haplotypes were CCTCGGCGCGCTAGAGCTGA (80%), CCTCGGCGCGCTAGCGCTGA (9.9%) and CCTCGGCGCGCGAGCGCTGA (3.5%).

Table 2.4.1 Genotype and allele frequencies of OCT1 *(SLC22A1)* gene SNPs in 130 healthy CA individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			
		Genotype	%	95% CI	Allele	%	95% CI	HWE (P)
S14F	rs34447885	CC	97.8	92.6 – 100.0	C	98.3	92.9 – 99.8	0.920
		CT	2.2	0.4 – 6.2	T	1.7	0.2 – 7.04	
		TT	0.0	0.0 – 3.1				
R61C	rs12208357	CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
		CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
C88R	rs55918055	TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0	
		TA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6	
		AA	0.0	0.0 – 1.3				
S189L	rs34104736	CC	98.1	92.6 – 100.0	C	98.1	92.3 – 99.9	0.920
		CT	1.9	0.4 – 6.2	T	1.9	0.2 7.0	
		TT	0.0	0.0 – 1.3				
G220V	rs36103319	GG	98.1	92.6 – 100.0	G	98.1	92.3 – 99.9	0.920
		GT	1.9	0.4 – 6.2	T	1.9	0.2 7.0	
		TT	0.0	0.0 – 1.3				
P283L	rs4646277	CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
		CT	0.0	0.0 – 1.3		0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
P341L	rs2282143	CC	96.2	92.6 – 98.9	C	98.6	92.3 – 99.9	0.998
		CT	3.8	0.4 – 6.2	T	1.4	0.2 7.0	

		TT	0.0	0.0 – 3.1				
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
G401S	rs34130495	GA	0.0	0.0 – 1.3	A	0.0	0.0 1.6	
		AA	0.0	0.0 – 1.3				
		GG	99.0	92.6 – 100.0	G	99.0	92.3 – 99.9	
M440I	rs35956182	GA	1.0	0.4 – 6.2	A	1.0	0.2 7.0	0.999
		AA	0.0	0.0 – 1.3				
		GG	99.0	92.6 – 100.0	G	99.0	92.3 – 99.9	
G465R	rs34059508	GA	1.0	0.4 – 6.2	A	1.0	0.2 7.0	0.999
		AA	0.0	0.0 – 1.3				
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
V519F	rs78899680	GT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
		AA	67.9	59.9 – 75.1	A	96.9	91.5 – 99.4	
Intronic SNP	rs622342	AC	30.1	21.2 – 39.9	C	3.1	0.6 – 8.5	0.871
		CC	2.4	0.4 – 6.2				
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
R287G	rs4646278	CG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6	
		GG	0.0	0.0 – 1.3				
I542V	rs137928512	AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0	
		AG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6	
		GG	0.0	0.0 – 1.3				
M420V	rs142448543	AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0	

		AG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6
		GG	0.0	0.0 – 1.3			
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0
A413V	rs144322387	CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
		AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0
I421F	rs151333280	AT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
C436F	rs139512541	GT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
		TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0
V501E	rs143175763	TA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
A306T	COSM164365	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			

Table 2.4.2 Comparison of MAF of *SLC22A1* gene SNPs of the CA population to other ethnic groups.

dbSNP ID	Amino acid change	Minor Allele	Minor Allele Frequency (%)							
			CA[a]	Luhya[b]	Yoruba[b]	African-American[c]	Japanese[d]	Chinese-Han[b]	Caucasian-Finish[b]	Caucasian-American[e]
rs34447885	S14F	T	1.0	2.6	1.7	3.1	0.0	0.0	0.0	0.0
rs12208357	R61C	T	0.0	0.0	0.0	0.0	0.0	0.0	5.4	7.2
rs55918055	C88R	A	0.0	0.0	0.0	ND	ND	0.0	0.0	0.6
rs34104736	S189L	T	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
rs36103319	G220V	T	1.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0
rs4646277	P283L	T	0.0	0.0	0.0	ND	ND	0.5	1.3	ND
rs2282143	P341L	T	0.5	8.0	9.0	8.2	16.8	12.4	16.7	0.0
rs34130495	G401S	A	0.0	0.0	0.0	0.7	0.0	0.0	1.6	1.1
rs35956182	M440I	A	0.5	0.0	0.0	0.5	0.0	0.0	2.7	0.0
rs34059508	G465R	A	0.5	0.0	0.0	0.0	0.0	0.0	1.1	4.0
rs78899680	V519F	T	1.5	2.0	6.0	ND	ND	0.0	0.0	ND
rs622342	Intronic	C	18.0	22.0	15.0	ND	ND	13.2	37.1	ND

This study; b. Data from 1000Genomes; c. Data from (Shu et al., 2003); d. Data from (Itoda et al., 2004)

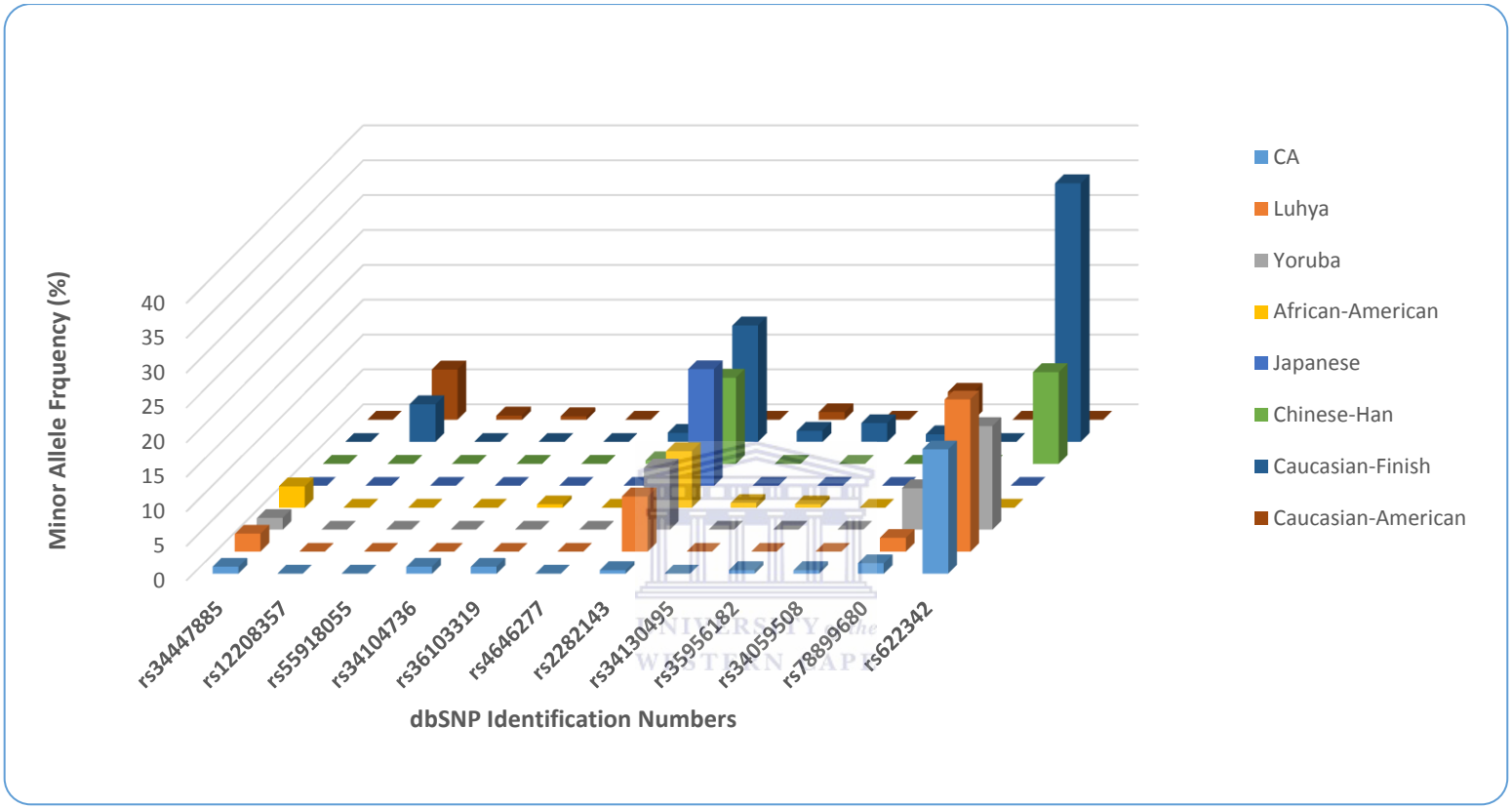


Figure 2.4.1. Allele frequencies of selected *SLC22A1* SNPs in the CA population compared to other African and world populations.

Table 2.4.3 Haplotype structure defined by 20 SNPs in the *SLC22A1* gene in the CA population.

Haplotype No.	Haplotype [a]	Frequency %
1	CCTCGGCGCGCTAGAGCTGA	80.0
2	CCTCGGCGCGCTAGCGCTGA	9.9
3	CCTCGGCGCGCGAGCGCTGA	3.5
4	CCTCGGCGGGCTAGAGCTGA	1.5
5	CCTCGGCGCGCTAGCGCTTA	1.1
6	TCTTTGCGCGCTAGCGCTGA	1.0
7	CCTCGGCGCGCTAGCGTTGA	0.6
8	CCTCGGAGCGCTAGAGCTGA	0.5
9	CCTCGGCGCGCGAGCACTGA	0.5
10	CCTCGGCGCGCTAGCGATGA	0.5
11	CCTCGGCGTGCTAGCGCTGA	0.5
12	CCTCGGCGCGCTAGCGTTTA	0.4
	TOTAL	100

a) Haplotype sequences are based on the position of SNPs on chromosome 6.

OCT2

The genotype and allele frequencies of the 20 investigated SNPs of the OCT2 gene within a group of 130 Cape Admixed subjects are summarized in **Table 2.4.4**. The allelic frequency of each SNP was in HWE ($p>0.05$). Sixteen of the eighteen investigated non-synonymous SNPs were monomorphic in the Cape Admixed population. Heterozygosity was only observed for the non-synonymous SNP variants R176H (rs57371881) and S270A (rs316019). The R176H variant genotype frequencies for homozygote wildtype (CC), heterozygote (CT) and homozygote (TT) were 91%, 9% and 0.0%. The MAF observed for R176H was 4.5%. Variant S270A displayed a genotype frequency of 90% homozygous (CC), 6% heterozygous (CA) and 4% homozygous (AA) for the Cape admixed population.

The MAF of a selected number of the investigated OCT2 gene SNPs in different ethnic groups are summarized in Table 8. Inferred haplotypes for each population are listed in **Table 2.4.5**. The most frequently observed haplotypes were CATAATGCGTACGCGCGACG (~85%), CATAATGATTACGCGCGACG (~7%) and CATAATGAGTACGCGCGACG (~4.5%).

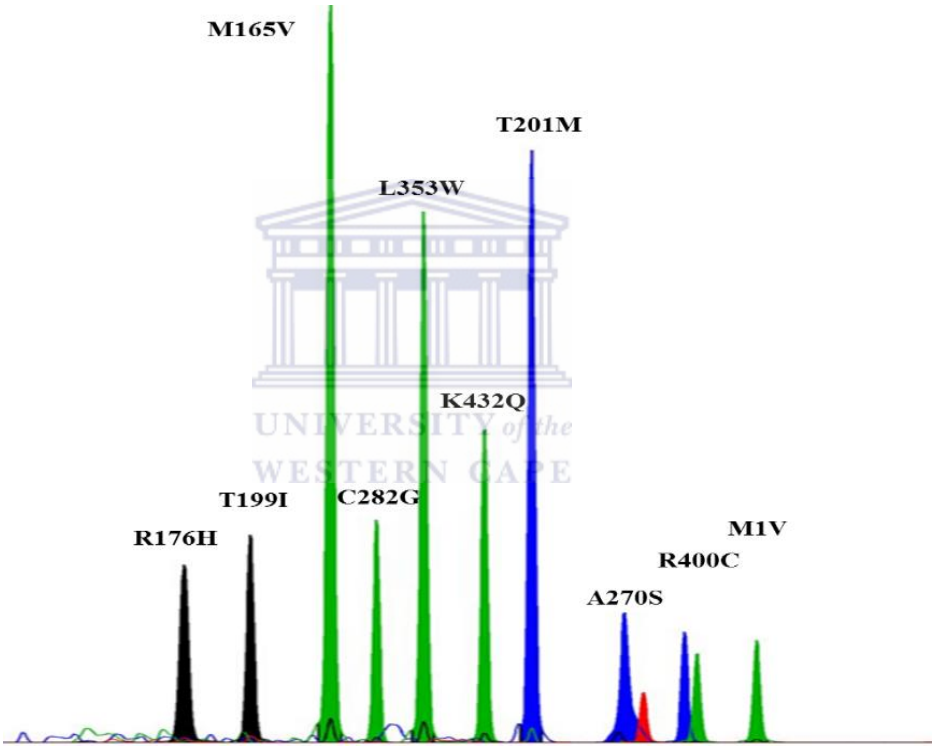


Figure 2.4.2. Typical electropherogram of *SLC22A2* multiplex 1 mini-sequencing fragments. Black represents C, green A, blue G, and red T.

Table 2.4.4 Genotype and allele frequencies of 20 known *SLC22A2* coding SNPs in 130 healthy Cape Admixed individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			
		Genotype	%	95% CI	Allele	%	95% CI	HWE (P)
M1V	rs141582772	CC	100.0	96.9 – 100.0	C	98.3	98.4 – 100.0	
		CT	0.0	0.0 – 1.3	T	1.7	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
M165V	rs8177508	TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0	
		TC	0.0	0.0 – 1.3	C	0.0	0.0 – 1.6	
		CC	0.0	0.0 – 1.3				
R176H	rs57371881	TT	91.3	85.4 – 96.6	T	92.2	86.7 – 97.3	
		TA	8.7	3.4 – 14.6	A	3.8	0.2 – 7.8	0.637
		AA	0.0	0.0 – 1.3				
T199I	rs201919874	CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
		CT	0.0	0.0 – 1.3	T	0.0	0.0 1.6	
		TT	0.0	0.0 – 1.3				
T201M	rs145450955	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
		GT	0.0	0.0 – 1.3	T	0.0	0.0 1.6	
		TT	0.0	0.0 – 1.3				
R207H	rs199783132	CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
		CT	0.0	0.0 – 1.3		0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
S270A	rs316019	CC	89.3	82.5 – 95.1	C	91.3	86.7 – 97.3	
		CA	6.7	2.0 – 12.0	T	7.4	2.7 13.3	0.655
		AA	4.0	0.2 – 7.8				

C282G	rs144729356	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
		GA	0.0	0.0 – 1.3	A	0.0	0.0 1.6
		AA	0.0	0.0 – 1.3			
L351W	rs45599131	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
		GA	0.0	0.0 – 1.3	A	0.0	0.0 1.6
		AA	0.0	0.0 – 1.3			
T357M	rs184227446	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
		GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
M393T	rs141205337	GG	100.0	88.6 – 96.9	G	100.0	98.4 – 100.0
		GT	0.0	0.0 – 1.3	T	3.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
R400C	rs8177516	CC	100.0	88.6 – 96.9	G	100.0	98.4 – 100.0
		CT	0.0	0.0 – 1.3	T	3.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
R404C	rs137885730	CC	100.0	87.8– 96.4	C	100.0	98.4 – 100.0
		CG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6
		GG	0.0	0.0 – 1.3			
K432Q	rs8177517	AA	100.0	88.6 – 96.9	G	100.0	98.4 – 100.0
		AC	0.0	0.0 – 1.3	T	3.0	0.0 – 1.6
		CC	0.0	0.0 – 1.3			
G439E	rs141405449	AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0
		AG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6
		GG	0.0	0.0 – 1.3			
R463K	rs3907239	CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0
		CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
R487Q	rs151282335	AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0
		AT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
R487W	rs140829992	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
		GT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
V502E/G	rs17853948	TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0
		TA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
V502M	rs140033522	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
		GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			

Table 2.4.5 Comparison of MAF of selected *SLC22A2* SNPs in the local populations to other ethnic groups.

dbSNP ID	Amino acid change	Minor Allele	Minor Allele Frequency (%)							
			Cape Admixed[a]	Luhya[b]	Yoruba[b]	African-American[b]	Japanese[b]	Chinese-Han[b]	Caucasian-Finish[b]	Caucasian-American[b]
rs8177508	M165V	C	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0
rs201919874	T199I	G	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0
rs145450955	T201M	A	0.0	0.0	0.0	0.0	2.2	0.5	0.0	0.0
rs199783132	R207H	T	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
rs316019	S270A	A	7.1	16.0	15.9	14.8	0.0	14.7	5.9	8.8
rs8177516	R400C	T	0.0	0.0	2.3	1.6	0.0	0.0	0.0	0.0
rs8177517	K432Q	G	0.0	5.2	6.2	1.6	0.0	0.0	0.0	0.0
rs57371881	R176H	T	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0

a. This study; b. Data from 1000 Genomes

Table 2.4.6 Haplotype structure of *SLC22A2* gene in the Cape Admixed population as defined by the 20 selected loci.

Haplotype Id	Haplotype	Frequency
Haplotype *1	CATAATGCGTACGCGCGACG	83.4%
Haplotype *2	CATAATGAGTACGCGCGACG	6.8%
Haplotype *3	CATAATGCGTACGCGCGACT	5.3%
Haplotype *4	TATAATGCGTACGCGCGACG	4.1%
Haplotype *5	TATAATGCGTACGCGCGACT	0.2%
Haplotype *6	TATAATGAGTACGCGCGACG	0.2%

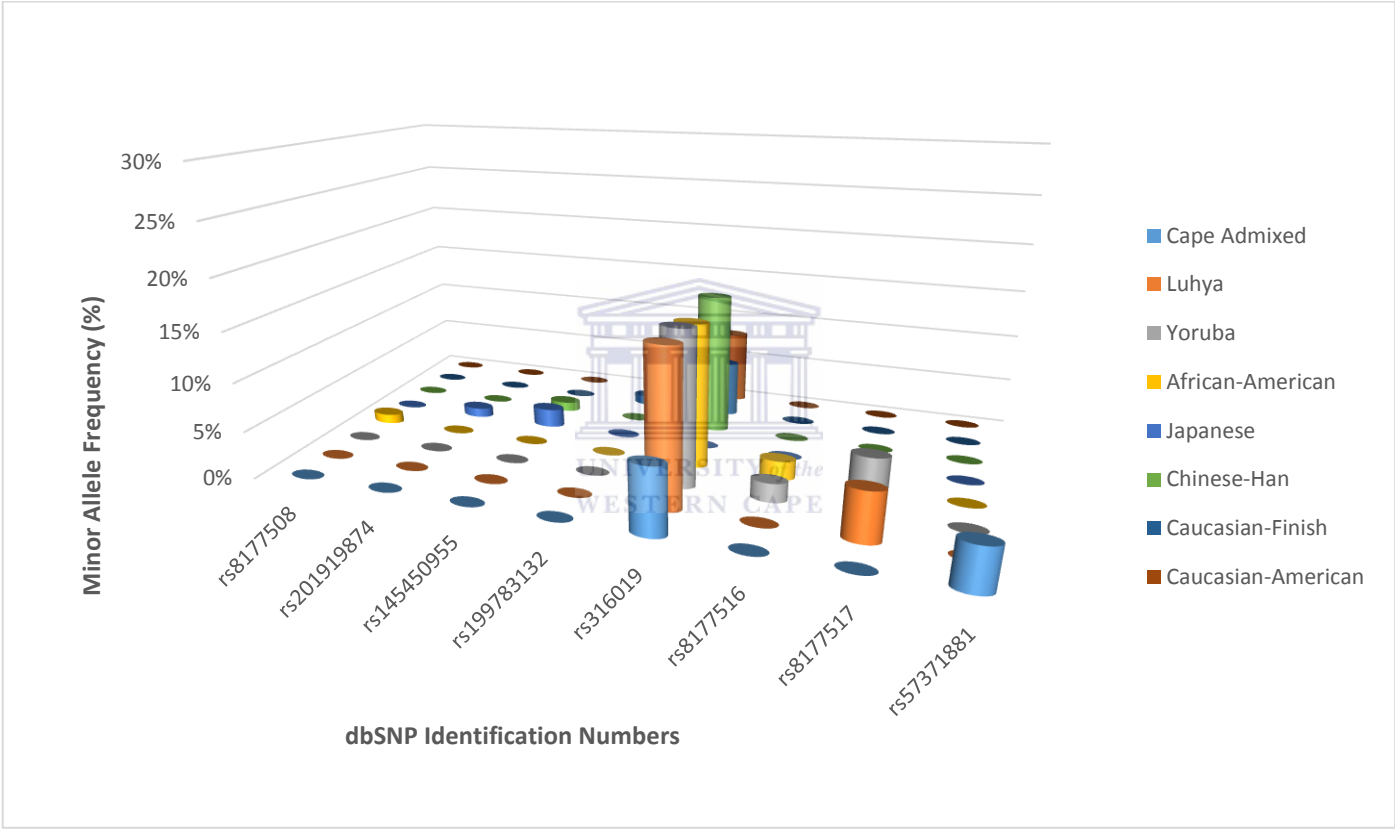


Figure 2.4.3. Allele frequencies of selected *SLC22A2* SNPs in the CA population compared to other African and world populations.

OCT3

In this study we have developed two SNaPshot™ multiplex assays for genotyping 18 known nonsynonymous coding SNPs in the *SLC22A3* gene. The genotype and allele frequencies of the 18 *SLC22A3* gene SNPs investigated in the 130 CA subjects are summarized in **Table 2.4.7**. All 18 coding SNPs genotyped in this study were monomorphic in the CA population.

Table 2.4.7 Genotype and allele frequencies of the OCT3 *(SLC22A3)* gene SNPs in 130 healthy Cape Admixed individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			HWE (P)
		Genotype	%	95% CI	Allele	%	95% CI	
N162I	rs139266499	AA AT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	A T	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
A169T	rs150004342	GG GA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	G A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
R212H	rs199688797	GG GA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	G A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
M248V	rs149101094	AA AG GG	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	A G	100.0 0.0	98.4 – 100.0 0.0 1.6	
G269E	rs147863404	GG GA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	G A	100.0 0.0	98.4 – 100.0 0.0 1.6	
R293C	rs142228053	CC CT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C T	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
R310C	rs183669984	CC CT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C T	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
S337F	rs141104413	CC CT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C T	100.0 0.0	98.4 – 100.0 0.0 1.6	
R348W	rs145328121	AA AT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	A T	100.0 0.0	98.4 – 100.0 0.0 1.6	
M370I	rs137958808	GG GT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	G T	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
I381T	rs187750009	TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0	

		TC	0.0	0.0 – 1.3	C	0.0	0.0 – 1.6
		CC	0.0	0.0 – 1.3			
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
V388M	rs189883656	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0
T400I	rs8187725	CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
R403H	rs200478210	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
R407H	rs145082363	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0
A439V	rs12212246	CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6
		TT	0.0	0.0 – 1.3			
		TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0
I431K	rs149424049	TA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0
R490Q	rs144856002	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6
		AA	0.0	0.0 – 1.3			

2.5. Discussion

2.5.1. OCT1

Single nucleotide polymorphisms in OCT1 gene have been recognized as a possible mechanism explaining inter-individual variation in drug response (Leabman et al., 2002b). In this study, we determined the genotypic and allelic frequencies of 20 SNPs in the OCT1 gene from 100 healthy individuals in the Cape Admixed population, and the data was compared to other population data available in literature and relevant databases.

In a study conducted by Leabman et al. (2002) numerous polymorphisms within the *SLC22A1* gene were identified, and have constituted the focus of many subsequent studies. Furthermore, it was determined that these variant might predict functional changes of the transporter protein (Leabman et al., 2002b). Several SNPs were found

to be protein-altering variants. This became important as these inherited differences may contribute to the inter-individual variability in drug response (Leabman et al., 2002b, Shu et al., 2008). Fifteen of these protein-altering SNPs were also characterized and evaluated with respect to MPP^+ uptake (Shu et al., 2008). It was determined that the variant S14F increased function of the transporter protein, whereas variants S189L, M420V and M440I were shown to exhibit the same functionality as the reference transporter. Furthermore, R61C and P341L were identified as reduced function variants, whereas variants G220V, G401S and G465R exhibited a loss of function compared to the reference sequence (Leabman et al., 2002b). In subsequent studies, by Shu et al (2008) and Tzvetkov et al (2009), evaluating the effect OCT1 variants have on metformin uptake, it was shown that the R61C, G401S, M420V and G465R variants all exhibited a reduction in transporter function. In a prior study by Kerb et al (2002), the uptake of 0.1 [3H]-1methy-l4-phenylpyridinium-([3H]MPP) by R61C, C88R and G401S were reduced to 30, 1.4 and 0.9% compared to wildtype, respectively. The transport of 1 µM [3H] serotonin by C88R and G401S, on the other hand, was reduced to only 13 and 12% of wildtype respectively (Kerb et al., 2002). From the data obtained, Kerb et al (2002) suggested that mutants R61C, C88R and G401S could affect the disposition of hOCT1 substrates in terms of affinity and selectivity. This has led them to conclude that the duration and intensity of effects of drugs and neurotransmitters may be altered for these substrates (Leabman et al., 2002b). In the present study, 19 protein-altering SNPs were evaluated within the Cape Admixed population. The intronic SNP (rs622342) was also included in the study because it was previously suggested to be associated with the blood glucose-lowering effect of metformin (Fujita et al., 2006).

Variant S14F was observed in the Cape Admixed, Luhya and Yoruba populations at a MAF of 1%, 2.5% and 1.5% respectively. This variant was not observed in any of the other populations included in the analysis (**Table 2.4.2**). Therefore, it may be suggested that OCT1 substrate drugs might have different response profiles in the Cape Admixed and African populations compared to Caucasian and Asian populations.

The G220V variant was observed in the Cape Admixed population with MAF of 1%, whereas, the other reduced-function variants G401S and G465R were not observed in the population. This may suggest an increased therapeutic response to metformin for our population (Becker et al., 2009a). The G401S and G465R variants were only observed in the Caucasian population at a MAF of 2.3% and 3.4% respectively. These variants were not observed in the African and Asian populations (**Table 2.4.2**).

The MAF of P341L observed in the Cape Admixed population (0.5%) was lower than that of other Sub-Saharan African, Asian and Caucasian populations (**Table 2.4.2**). Functional *in vitro* transport assays have shown that the P341L variant results in a decrease rate of MPP+ transport, and has no effect on the transport of the antidiabetic drug metformin (Becker et al., 2009a, Crawford and Nickerson, 2005)

The observed MAF at loci S189L, M440I and M420V was 1%, 0.5% and 4% respectively. However, S189L was only observed in the Cape Admixed population whereas M440I was observed in the Cape Admixed and Native American/Hispanic Admixed populations at a similar frequency (**Table 2.4.2**). Although at low frequencies, these variants are suggested to have a reduced transporter function with respect to metformin (Fujita et al., 2006).

The observed MAF for variant V519P in our population was 1.5%. This value was lower than that of the Luhya and Yoruba, with respective values of 2% and 6% (Table 2.4.2). However, this variant was not observed in any of the other populations, indicating that it might be specific to African populations. The impact of this variant on transport function or drug efficacy has not yet been determined and requires further investigation.

The intronic SNP (rs622342) was observed in the Cape Admixed population at lower MAF (18%) compared to previously reported frequencies for the European population (**table 2.4.2**). This includes the Dutch diabetic patients cohort where the variant was initially reported (Becker et al., 2009a). In this population-based cohort study, Becker *et al* (2009) concluded that an association existed between genetic variation in the gene encoding for the OCT1 transporter protein and the glucose lowering effect of metformin in diabetes mellitus patients, and that metformin therapy was less effective in patients carrying the minor C allele.

Twelve out of the nineteen investigated non-synonymous SNPs were monomorphic in the study population. No polymorphisms were observed for these SNPs and they were all homozygous for the ancestral allele. This includes variants R61C, C88S, P283L, R287G, A306T, G401S, A413V, M420V, I421F, C436F, V501E and I542V. The population data available in the databases and the literature for variants R61C, C88S, P283L and G401S is limited, and low MAFs are usually reported for these SNPs. To our knowledge, this study is the first to report population data for A306T; A413V; M420V, C436F; I421F: V501E, V519F and I542V. Further investigations are needed to explore the genetic variations among these SNPs in world populations.

The haplotype structure defined by 20 SNPs in the *SLC22A1* gene was also inferred for the Cape Admixed population. A total of 12 haplotypes were observed in the

study population (**Table 2.4.3**). Haplotype structure could provide a more complete picture of genetic variations at multiple loci important in drug response at the individual and population level (Crawford and Nickerson, 2005).

In our study population, it could be interesting to investigate the overall effect of the observed rare haplotype on the transport efficiency of the OCT1 protein, as well as possible clinical implications related to therapeutic drugs including metformin. This rare haplotype includes variants S14F, S189L, G220V and the intronic SNP rs622342, where further investigations are required to determine the cumulative effect of these variants on substrate transport efficiency and glucose-lowering effect of metformin.

2.5.2. OCT2

Drug transporters modulate the absorption, distribution, and elimination of drugs by controlling the influx and efflux of drugs in cells. Increasing evidence indicates genetic polymorphisms of transporters can have profound impact on drug disposition, drug efficacy, and drug safety (Ma and Lu, 2011). Single nucleotide polymorphisms in OCT2 gene have been recognized as a possible mechanism explaining inter-individual variation in drug response (Leabman et al., 2003b). In this study we compared the genotypic and allelic frequencies of 20 SNPs in the OCT2 gene from 140 healthy individuals in the Cape Admixed population.

In cellular studies, the variant S270A has been shown to variably alter the transport function of OCT2. (Leabman et al., 2002b) showed that the S270A variant had a Km value for MPP^+ uptake which was not significantly different from that of the OCT2 reference in oocytes. (Kang et al., 2007b) reported that the uptakes of MPP^+ and TEA were about 40% lower in MDCK cells transiently transfected with

OCT2A270S variant than that in MDCK cells transiently transfected with reference OCT2. (Choi et al., 2012) determined that this variant decreased uptake of MPP^+, TEA and Lamivudine in oocytes when compared with the wild type. Furthermore, it has been shown to decrease metformin transport activity and renal (tubular) clearance (Song et al., 2008b, Wang et al., 2008b). (Li et al., 2010) determined that individuals carrying the AA-genotype were not able to effectively bind metformin and excrete it via urine, thereby increasing the blood concentration of metformin and decreasing renal uptake of the drug. The S270A variant was observed in the CA population with a MAF of 7%which is lower than the average MAF of 12% for global populations (**Table 2.4.5**).

Interestingly, no data exists in the public domain on variant R176H. It should be noted that further investigation into the effect this variant has on protein regulation and expression is required and, therefore, should be included in future transport and efficacy assays.

In this study the 20 investigated nonsynonymous *SLC22A2* SNPs showed a low level of heterozygosity, with roughly 95% of the loci displaying monomorphism (**Table 2.4.4**). This study once again highlighted the fact that the distribution of SNPs and the MAFs thereof differ between the Cape Admixed and other populations, and even amongst African populations themselves. These differences in allele frequencies, although not fully understood, might provide us with the insight into interethnic and inter-individual variability in drug response for substrate drugs of hOCT2. Moreover, the single SNPs identified and haplotypes inferred for these populations may be important in future pharmacogenomic in identifying association between causative variants and altered drug response.

2.5.3 OCT3

In the current study we genotyped 18 known SNPs in the *SLC22A3* gene of 130 CA individuals residing in the Cape Town metropolitan area, South Africa. We observed no genetic variation for the 18 noncoding SNPs genotyped in the investigated population (**Table 2.4.7**). However, this lack of genetic variation in the coding region of *SLC22A3* in the CA population is not a unique situation and has also been observed in other populations (Kang et al., 2007a, Lazar et al., 2003). Moreover, according to Lazar *et al.* (2008), this high degree of genetic preservation and lack of protein sequence variation may reflect the crucial physiological role hOCT3 plays in maintaining homeostasis (Lazar et al., 2008).

Unlike its paralogues hOCT3 has a broad distribution in the human body and because of its localization in the CNS and its affinity for monoamines is believed to play a significant role in neuropsychiatric disorders. Methamphetamine (MAP) is a powerful highly addictive psychostimulant that affects the CNS. The illicit us of MAP has become a growing problem in a number of countries over the last two decades, and has recently emerged as a significant problem in South Africa (Plüddemann et al., 2010). Smoking crystalline MAP has been associated with high levels of harm. The most salient harms associated with MAP use are mental health problems, including psychosis, depression, anxiety, and violent behaviour. Ayoma *et al.* (2006), in a study with Japanese MAP users, found that *SLC22A3* polymorphisms may be related to the development of polysubstance use in patients with MAP dependence (Aoyama et al., 2006). The polymorphisms used in the Aoyama study

were not included in the present study and as such no inferences can be drawn. However, given the health problems associated with, and the current prevalence of MAP use in South Africa, the role of genetic polymorphisms of *SLC22A3* in the development of polysubstance use in individuals with MAP dependence in indigenous African populations need further investigation.

The role of hOCT3 in monoamine transport *in vivo* is well established. Because of its role in monoamine transport and its localization in the CNS, hOCT3 has also been implicated in non-neuronal termination of noradrenergic signalling in the CNS and as a candidate gene for a variety of neuropsychiatric disorders (Lazar et al., 2008, Wultsch et al., 2009). Moreover, Lazar *et al.* (2008) identified two SNPs, 106/107delAG and Met370Ile in a group of Caucasian patients diagnosed with obsessive compulsive disorder (OCD), which lead to a decrease in promoter activity and transport of norepinephrine, respectively, in assays performed *in vitro* (Lazar et al., 2008). Haenisch *et al.* (2012) showed that two psychoactive drugs nefazodone and clozapine had the potential to inhibit hOCT3 activity. However, their study was unable to demonstrate whether this direct inhibition of hOCT3 plays a role in the clinical effects of these drugs (Haenisch et al., 2012).

The ubiquitously expressed hOCT3 has not only been implicated in the transport of anticancer drugs, but more recently also as a biomarker for cancer pathogenesis. For example, in colorectal cancers hOCT3 together with its paralogues, hOCT1 and hOCT2, have been shown to be determinants of oxaliplatin cytotoxicity (Yokoo et al., 2008, Zhang et al., 2006). Moreover, *SLC22A3* expression in renal cell carcinoma cell lines enhances the sensitivity of these cell lines towards the chemotherapeutic agents melphalan, irinotecan, and vincristine (Shnitsar et al.,

2009). Cui *et al.* (2011) recently identified the *SLC22A3* SNP rs7758229 as a risk locus for distal colon cancer in an Asian population (Cui et al., 2011). In addition, a study by Grisanzio *et al.* (2012) showed that *SLC22A3* is inversely correlated with prostate cancer progression, with markedly decreased expression in aggressive prostate cancers (Grisanzio et al., 2012).

Metformin is a biguanide antidiabetic drug and is widely used as a first-line therapeutic in the treatment of type2 diabetes. Earlier studies have shown that metformin is transported by hOCT1 and hOCT2, and that genetic polymorphisms of these transporters affect the pharmacokinetic and therapeutic effect of the drug (Becker et al., 2009a, Becker et al., 2010, Kimura et al., 2009, Shu et al., 2007, Shu et al., 2008). Recent studies have now also implicated hMATEs and hOCT3 in metformin absorption, disposition, and pharmacological action (Becker et al., 2010, Chen et al., 2010a). Chen et al (2010) found that the hOCT3 variant T400I significantly reduced metformin uptake by the transporter (Chen et al., 2010a). Structural modelling suggested that this variant may be located in the pore lining of the TMHs, where it plays a critical role in substrate translocation. The T400I variant is a rare variant that has a low allele frequency and was not observed in the individuals that participated in this study. Given the prevalence of type2 diabetes in South Africa and the widespread use of metformin as a therapeutic, the distribution of this variant in the indigenous African populations require further investigation. The effect of this variant *in vivo* on metformin pharmacokinetics and efficacy has not been demonstrated yet, but should be assessed if the T400I variant is identified in the CA or any of the indigenous African populations.

Ideally a larger sample size and complete sequencing of the *SLC22A3* gene would provide a more complete picture of the spectrum of genetic variation within this gene for the Cape Admixed population. In addition, a number of SNPs in the proximal promoter region had been associated with altered expression of the *SLC22A3* gene previously, however the current approach of genotyping coding SNPs only, excluded these variants from being assayed. Furthermore, although medical research has primarily focused on protein-coding variants, this picture has changed with advances in the systematic annotation of functional noncoding elements (Ward and Kellis, 2012). However, the genotyping strategy adopted in this study have excluded the typing of noncoding SNPs, which could be useful when performing linkage disequilibrium analysis or extracting information about disease association.

2.6. Conclusions

To our knowledge, this is the first study that investigated the allele and genotype frequency distributions of SNPs in the *SLC22A1-3* genes of the CA population. This study also reports the observed haplotypes in the investigated population. It has also been shown that reduced-function nonsynonymous SNPs in the *SLC22A1-3* genes found in Caucasian and Asian populations are absent from the CA population. We have shown that, although MAF observed for the CA population is largely similar to other African populations, differences exist that may translate into differences in organic cationic drug transport between these ethnic groups. These variations may translate into differences in the transport and efficacy of organic cationic drugs commonly used for the treatment of diseases prevalent in Africa. However, it should be noted that this was only a descriptive study and that no associations are made between any diseases or treatment outcomes. Although, it is well known that

individual variation in drug response can be attributed to specific genetic variants. Moreover, it is believed that the incorporation of haplotypes in pharmacogenomic studies will provide a more complete picture of loci that are relevant in the practice of "genetic medicine" both at an individual or population level (Crawford and Nickerson, 2005). This study contributes towards filling the gap that exists with regards to genetic information about important variations in organic cation transporter genes, such as *SLC22A1-3*, for the indigenous populations of South Africa given that organic cationic drugs are used in the treatment of diseases such as type-2 diabetes mellitus, various cancers, and HIV, these variants may impact profoundly on healthcare provided over the African continent. Therefore, given the aforementioned reasons studies such as this is valuable in the generation of useful pharmacogenomic information specific for Admixed populations.

CHAPTER 3

Assessment of Genetic Variations within the *SLCO1B1* Gene of the Cape Admixed Population

3.1. Abstract

Human organic anion transporter OATP1B1 plays an important role in the transport of a wide variety of substrates. It includes bile salts, steroid conjugates cyclic peptides, drugs such as benzylpenicillin, methotrexate, pravastatin and rifampicin, and natural toxins microcystin and phalloidin. Numerous polymorphisms have been discovered in the *SLCO1B1* gene. This includes RS4149056 and RS2306283, two of the most characterized SNPs in this gene. A SNaPshot® mini-sequencing panel was designed and optimized to investigate genetic variants in admixed and indigenous populations. The minor allele frequencies (MAF) of genetic variants observed were compared between the local populations and world populations. Possible clinical implications were determined. The observed MAFs for the coding region *SLCO1B1* variants V174A (rs4149056), L191L (rs4149057), P155T (rs11045819), and F199F (rs2291075) were 60.0%, 47.5%, 21.5%, 16%, respectively, for the CA population. The observed MAFs in the CA population for the intronic variants Rs4149032 and Rs4149081 were 36% and 8.3%, respectively. The most frequently observed haplotypes were TATCGACGACCCGATGTGAT (10.8%), CATCGACGACCCGATGTGAT (9.1%), TACCGACGACCCGATGTGAT (7.9%).

The mixed ancestry can potentially provide the intrinsic variability needed to unravel intricate gene-environment interactions, which may help explain the population differences in the epidemiology of complex disease.

CHAPTER 3

3.2. Introduction

OATP1B1 is encoded by the *SLCO1B1* gene, located on chromosome 12 (12p12). OATPs are sodium-independent uptake transporters that mediate the influx of a variety of amphipathic compounds. Their transport mechanism is electroneutral exchange, coupling the cellular uptake of organic compounds with the efflux of neutralizing anions such as bicarbonate, glutathione and glutathione-S-conjugates (Li et al., 1998, Satlin et al., 1997). The majority of OATPs transport a wide assortment of amphipathic organic compounds (Bossuyt et al., 1996). OATP1B1 is expressed at the basolateral plasma membrane of hepatocytes (Abe et al., 2001) and has a total mass of 84kDa, which is reduced to 54kDa after de-glycosylation (Konig et al., 2000). Since it is primarily expressed in the human liver it is suggested that OATP1B1 plays a critical role in the hepatic clearance of albumin-bound amphipathic compounds (Hagenbuch and Meier, 2004). This includes bile salts, conjugated and unconjugated bilirubin, BSP, steroid conjugates, thyroid hormones T4 and T3, eicosanoids, cyclic peptides, drugs including benzylpenicillin, methotrexate, pravastatin and rifampicin as well as natural toxins such as mircosystin and phalloidin (Hagenbuch and Meier, 2003).

Numerous synonymous and nonsynonymous SNPs have been discovered on the *SLCO1B1* gene. Many of these variants have been shown to affect transport function, both *in vitro* and *in vivo* (Iwai et al., 2004, Tirona et al., 2001). Furthermore, it is recognized that the majority of these SNPs span the transmembrane domain or extracellular loop 5 of OATP1B1 (Iwai et al., 2004, Tirona et al., 2001). This includes RS4149056 and RS2306283, the most well characterized SNPs in this gene, which produce amino acid changes Val174Ala and Asn130Asp respectively. However, when the effects of particular SNPs are evaluated it is important to take

into account the underlying haplotype these SNPs are found in (Mwinyi et al., 2008). For example, the abovementioned SNPs are in linkage equilibrium and exist in varying haplotypes together. However, when the nucleotide Adenine (A) is present at RS2306283 and Thymine (T) is present at RS4149056 it is referred to as haplotype *1A or the reference haplotype. Furthermore, SNPs in the promoter region of this gene can further sub-classify its haplotype into two distinct functional haplotypes (Niemi et al., 2004). Although, it is important to note that the effects of certain *SLCO1B1* SNPs on transport function are substrate-specific (Tirona et al., 2001).

Haplotypes *5 and *15 have been associated with a strikingly reduced uptake of multiple OATP1B1 substrates including oestrone3sulphate, oestradiol17βD-glucuronide, atorvastatin, cerivastatin, pravastatin and rifampicin (Iwai et al., 2004, Kameyama et al., 2005, Nozawa et al., 2005, Tirona and Kim, 2005, Tirona et al., 2001). Additionally, RS4149056>C has been associated with increased plasma concentrations of many statins as well as fexofenadine and repaglinide (Chung et al., 2005, Lee et al., 2005, Mwinyi et al., 2008, Niemi et al., 2005a, Niemi et al., 2005b, Niemi et al., 2004, Nishizato et al., 2003). It is important to note that these haplotype designations only refer to the SNPs in linkage disequilibrium. Other, less studied SNPs also occur in the SLCO1B1 gene which are potentially functional variants.

Sub-Saharan Africa, and for that matter South Africa, has a significant disease burden of both communicable and non-communicable diseases (Coovadia et al., 2009, Mayosi et al., 2012). The current belief is that even developing nations, such as those in sub-Saharan Africa, can benefit from pharmacogenomics in order to inform public health policies, designing and interpreting clinical trials, and possibly

to help guide clinicians to prospectively evaluate those patients with the greatest probability of expressing a variant genotype (Daar and Singer, 2005b, Suarez-Kurtz, 2008a).

Although African populations harbour more genetic diversity than Caucasians, for example, this genetic diversity is however understudied (Hardy et al., 2008). This lack of genetic information with robust allele frequencies currently serves as a significant hurdle to designing biomedical research and medical implications (May et al., 2013). South Africa is home to several indigenous African populations for which there is limited or no genomic information. The Cape Admixed (CA) population, historically indigenous to the Western Cape Province, is the second largest ethnic group in South Africa, and comprises approximately 9% (~4 million) of the total population (Drögemöller et al., 2010). Previous studies have shown that these populations harbour unique genotype and allele frequencies for pharmacogenomically relevant drug metabolizing enzymes (Ikediobi et al., 2011). However, to our knowledge only a limited number of studies have to date been undertaken to establish baseline genotype and allele frequency distributions of genetic polymorphisms in membrane transporter genes of admixed South African populations. Therefore, the aim of this study was to determine the baseline genotype and allele frequency distributions of 20 known *SLCO1B1* coding SNPs in 130 CA individuals residing in Cape Town, South Africa

3.3. Materials and Methods

3.3.1. Subjects

Subjects were as described in **Chapter 2**.

3.3.2. DNA extraction and SNP selection

A standard salt lysis method was used for the isolation of genomic DNA was isolated from buccal swab samples as described in **Chapter 2** and stored frozen at 20°C until the time of genotyping (Leat et al., 2004). A total of 20 *SLCO1B1* coding SNPs were selected for this study. SNPs were selected from the literature and the Ensembl database (http://www.ensebl.org) (Flicek et al., 2012). Variants, were included in this study based on predicted effect on function, using the SIFT (Sorting Intolerant From Tolerant) program (Flanagan et al., 2010, Kumar et al., 2009, Ng and Henikoff, 2003). To our knowledge no population data exist in the public domain for these variants.

3.3.3. Primer design

Multiplex PCR primers for the amplification of all *SLCO1B1* exons and flanking regions were designed using Primer3 software (www.genome.wi.mit.edu/cgibin/primer/primer3) and are listed in **Table 3.3.1**. To test for possible nonspecific amplification, primers were aligned with the NCBI sequence databases using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast/blastcgi). Two SNaPshot™ Multiplex systems were specifically designed for the study, successfully optimized and used for genotyping. The single base extension primer sets for multiplex 1 and 2 are listed in **Tables 3.3.2** and **3.3.3**.

3.3.4. Multiplex PCR

All the required *SLCO1B1* exons and their flanking regions were simultaneously amplified using the primers listed in **Table 3.3.1**. The PCR reactions were performed in a 20 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qiagen multiplex

PCR master mix (Qiagen, Courtaboeuf, France) and 0.2 μM of each primer. Cycling parameters used and PCR product purification were as described in **Chapter 2**.

3.3.5. SNaPshot™ genotyping reactions

Multiplex mini-sequencing was performed in a 10 μl reaction volume using 3 μl of a 1/10 dilution of purified PCR products, 0.10.2 μM of primers, and 5 μl of SNaPshot™ ready reaction mix (Also observed in the promoter region within the Xhosa population (Jacobs et al., 2015)). Sequence cycling was performed according to the instructions of the manufacturer. Post-extension treatment was done as described in **Chapter 2.**

3.3.6. Electrophoresis of the mini-sequencing products

The fluorescently labelled fragments were treated and separated on an ABI Prism 3500 Genetic Analyzer (Also observed in the promoter region within the Xhosa population (Jacobs et al., 2015)) as described in **Chapter 2**. Data analyses were performed using GeneMapper® IDX Software Version 1.2.

Table 3.3.1 *SLCO1B1* multiplex PCR primers for the amplification of exons and flanking regions.

Location	(Forward primer 5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)
Exon 5	GGGGAAGATAATGGTGCAAA	TGTTGTTAATGGGCGAACTG	403
Exon 67	TCCTTTATAAATTACCCAGTCTCAGG	GATCCCAGGGTAAAGCCAAT	618
Exon 9	GCTGTGAACAGCCTGTGGTA	TGCAACTTCAAATGCAGAGC	516
Exon 10	TGATAGGTGCAGCAAACCAC	TTGCTTCTCTTTAGTGGAGGAAT	598
Exon 11	AAAAACTTTGCCATTTCGTCA	TTTGTTTATACATCACACCCATCA	501
Exon 13	TAATGGGGCCATTCAACTGT	GGTCCATCACTCAATTTTACTCTG	417
Exon 15	TCGTTATGCCCCAATAAAAA	TGGAATGTCTTTATTTCTTCCACA	962
Intron 1	TAGGGGCTTTTCATGTGCTT	GGCAGGTACAGAGGAACAGG	304
Intron 2	CATTTTGCTGCAACCATATCA	ACCTTGGACATTAAGCTCTCTTC	321
Intron 3	CTGACTTTGCATGCAGTATGG	GAGAAGTCAGAGGCAATCGAA	300
Intron 4	AGGGATATTGGCCTGTTGGT	CCATCCAAAATGAACCATCC	328
Promoter	AAGCCATTCCCAAAAGTAAGG	AAGCGTGTGGAAGACACAGA	371

Table 3.3.2 Multiplex 1 of *SLCO1B1* single-base extension primers for SNaPshot™ mini-sequencing reactions.

NCBI (dbSNP)	Amino Acid Change	Nucleotide change	Nucleotide sequence (5′ to 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	Poly-GACT tail
rs4149056	V174A	C/T	aggaatctgggtcatacatgtggatatatg	14093965	30	0
rs2306283	N130D	A/G	ataaggtcgatgttgaattttctgatgaat	14092154	35	5
rs4149057	L191L	C/T	tagcgaaatcatcaatgtaagaaagcccca	14094015	40	10
rs57040246	Y362Y	C/T	agatgaaggctgaccatactgttgctctac	14115973	45	15
rs59502379	G488A	C/G	gaataacttacatctcaccctgtctagcag	14121349	50	20
rs34671512	L643F	A/C	agtctcatcacttgttttatatattatatt	14154392	55	25
rs11045819	P155T	A/C	aatcaaattttatcactcaatagagcatca	14092229	60	30
rs11045818	S137S	A/G	aatttgattaattaaacaagtggataaggt	14092177	65	35
rs79135870	I222V	A/G	tagaaaacagagatcccagggtaaagccaa	14094307	70	40
rs2291075	F199F	C/T	accattggggctttcttacattgatgattt	14094041	75	45

Table 3.3.3 Multiplex 2 of *SLO1B1* single-base extension primers for SNaPshot™ mini-sequencing reactions.

NCBI (dbSNP)	Amino Acid Change	Nucleotide change	Nucleotide sequence (5′ to 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	Poly-GACT tail
rs61760243	T345M	C/T	atcccctgtatgttatgtttgtgcttttga	14115921	30	0
rs59113707	F400L	C/G	gaatttggcaattccaacggtgttcagttt	14117905	35	5
rs11045859	V416V	A/G	aattgccaaattctcatgttttactgctgt	14117953	40	10
rs74700754	H575L	A/T	ctgaattgaaatcacttgcactgggtttcc	14137691	45	15
rs4149032	Intron	C/T	gtccctttttcctatctgcacataacatta	14080207	50	20
rs4149081	Intron	A/G	tagccattttctattatctctgatttttg	14140437	55	25
rs4363657	Intron	C/T	tttttcagaataatttagtacagtgggtac	14131138	60	30
rs4149015	Intron	A/G	cacatatatatatgtgcatatgtgtataca	14045738	65	35
rs59710386	Intron	A/C	aacctagttagttttaagagttcactccag	14046426	70	40
rs4149087	Intron	G/T	caaacaaacacagagtttgaactataatac	14154978	75	45

3.4. Results

Our study population consisted of a 130 healthy, unrelated CA individuals residing in the Cape Town Metropolitan area, South Africa. The age of the participants ranged from 18 to 60 years with the mean age of female participants being 25.3±9.0 years, while male participants had a mean age of 24.8±7.7 years. There were 80 (54%) female and 68 (46%) male participants.

In this study we have developed two SNaPshot™ multiplex assays for genotyping 20 known nonsynonymous coding SNPs in the *SLCO1B1* gene. The genotype and allele frequencies of the 20 *SLCO1B1* gene SNPs investigated in the 130 Cape Admixed subjects are summarized in **Table 3.4.1**. The allelic frequency of each SNP was in HWE ($p>0.05$). Fourteen of the nineteen investigated nonsynonymous SNPs were monomorphic in the CA population.

The observed MAFs for the coding region *SLCO1B1* variants V174A (rs4149056), L191L (rs4149057), P155T (rs11045819), and F199F (rs2291075) were 60.0%, 47.5%, 21.5%, 16%, respectively, for the CA population. The observed MAFs in the CA population for the intronic variants Rs4149032 and Rs4149081 were 36% and 8.3%, respectively. Inferred haplotypes for each population are listed in **Table 3.4.2**. The most frequently observed haplotypes were TATCGACGACCCGATGTGAT (10.8%), CATCGACGACCCGATGTGAT (9.1%), TACCGACGACCCGATGTGAT (7.9%), and CACCGACGACCCGATGTGAT (7.7%).

Table 3.4.1 Genotype and allele frequencies of OATP1B1 *(SLCO1B1)* gene SNPs in 130 healthy CA individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			
		Genotype	%	95% CI	Allele	%	95% CI	HWE (P)
		TT	25.0	16.5 –33.5	T	40.0	30.4 – 49.6	
V174A	rs4149056	TC	30.0	21.0 – 39.0	C	60.0	0.2 – 7.04	14.063
		CC	45.0	25.7 – 44.3				
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
N130D	rs2306283	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6	
		AA	0.0	0.0 – 1.3				
		TT	52.0	42.2 – 61.8	T	52.4	42.2 – 61.8	
L191L	rs4149057	TC	0.8	0.01 – 3.0	C	47.6	37.2 – 56.8	96.030
		CC	47.2	37.2 – 56.8				
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
Y362Y	rs57040246	CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
G488A	rs59502379	GC	0.0	0.0 – 1.3	C	0.0	0.0 – 1.6	
		CC	0.0	0.0 – 1.3				
		AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0	
L643F	rs34671512	AC	0.0	0.0 – 1.3	C	0.0	0.0 – 1.6	
		CC	0.0	0.0 – 1.3				
P155T	rs11045819	CC	96.2	92.2 – 99.8	C	78.5	71.0 – 87.0	4.005

		CA	3.8	0.2 – 7.8	A	21.5	13.0 29.0	
		AA	0.0	0.0 – 3.1				
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
S137S	rs11045818	GA	0.0	0.0 – 1.3	A	0.0	0.0 1.6	
		AA	0.0	0.0 – 1.3				
		AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0	
I222V	rs79135870	AG	0.0	0.0 – 1.3	G	0.0	0.0 1.6	
		GG	0.0	0.0 – 1.3				
		TT	3.0	0.3 – 6.3	T	16.0	8.8 – 23.2	
F199F	rs2291075	TC	26.0	17.4 – 34.6	C	84.0	76.8 91.2	0.107
		CC	71.0	62.1 – 79.9				
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
T345M	rs61760243	CT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				
		CC	100.0	96.9 – 100.0	C	100.0	98.4 – 100.0	
F400L	rs59113707	CG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6	
		GG	0.0	0.0 – 1.3				
	rs11045859	GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
V416V		GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6	
		AA	0.0	0.0 – 1.3				
H575L	rs74700754	AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0	
		AT	0.0	0.0 – 1.3	T	0.0	0.0 – 1.6	
		TT	0.0	0.0 – 1.3				

	rs4149032	TT	52.0	42.2 – 61.8	T	64.0	56.4 – 73.4	
Intronic SNP		TC	24.0	15.6 – 32.4	C	36.0	26.6 – 45.4	22.960
		CC	24.0	15.6 – 32.4				
		GG	90.7	85.4 – 96.6	G	90.7	85.4 – 96.6	
Intronic SNP	rs4149081	GA	0.0	0.0 – 1.3	C	9.3	3.4 – 14.6	100.00
		AA	9.3	3.4 – 14.6				
		TT	75.0	66.5 – 83.5	T	85.5	79.2 – 92.8	
Intronic SNP	rs4363657	TC	21.0	13.0 – 29.0	C	14.5	7.2 – 20.8	
		CC	4.0	0.2 – 7.8				
		GG	100.0	96.9 – 100.0	G	100.0	98.4 – 100.0	
Intronic SNP	rs4149015	GA	0.0	0.0 – 1.3	A	0.0	0.0 – 1.6	
		AA	0.0	0.0 – 1.3				
		AA	100.0	96.9 – 100.0	A	100.0	98.4 – 100.0	
Intronic SNP	rs59710386	AC	0.0	0.0 – 1.3	C	0.0	0.0 – 1.6	
		CC	0.0	0.0 – 1.3				
		TT	100.0	96.9 – 100.0	T	100.0	98.4 – 100.0	
Intronic SNP	rs4149087	TG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6	
		GG	0.0	0.0 – 1.3				

Table 3.4.2 Comparison of MAF of *SLC22A1* gene SNPs of the CA population to other ethnic groups

dbSNP ID	Amino acid change	Minor Allele	Minor Allele Frequency (%)						
			CA[a]	Luhya[b]	Yoruba[b]	Peurto Rican[b]	Japanese[b]	Chinese-Han[b]	Caucasian-British[b]
rs4149056	V174A	C	60.0	2.0	1.0	12.0	12.0	12.0	14.0
rs2306283	N130D	A	0	16.0	19.0	47.0	34.0	20.0	64.0
rs4149057	L191L	C	47.5	5.0	15.0	50.0	34.0	21.0	64.0
rs57040246	Y362Y	T	0	3.0	7.0	1.0	0	0	0
rs59502379	G488A	C	0	5.0	5.0	0	0	0	0
rs34671512	L643F	C	0	7.0	6.0	8.0	0	0	4.0
rs11045819	P155T	A	21.5	3.0	5.0	14.0	0	0	15.0
rs11045818	S137S	A	0	0	0	12.0	0	0	15.0
rs79135870	I222V	G	0	1.0	3.0	0	0	0	0
rs2291075	F199F	T	16.0	55.0	60.0	41.0	38.0	51.0	35.0
rs59113707	F400L	G	0	3.0	7.0	1.0	0	0	0
rs11045859	V416V	A	0	3.0	12.0	1.0	0	0	0
rs4149032	Intron	C	36.0	28.0	28.0	56.0	49.0	39.0	66.0
rs4149081	Intron	A	9.0	19.0	16.0	18.0	38.0	45.0	15.0
rs4363657	Intron	C	14.5	17.0	13.0	18.0	38.0	45.0	15.0

rs4149015	Intron	A	0	3.0	0	4.0	12.0	12.0	2.0
rs59710386	Intron	C	0	0	0	2.0	0	0	3.0
rs4149087	Intron	G	0	31.0	27.0	39.0	71.0	79.0	38.0

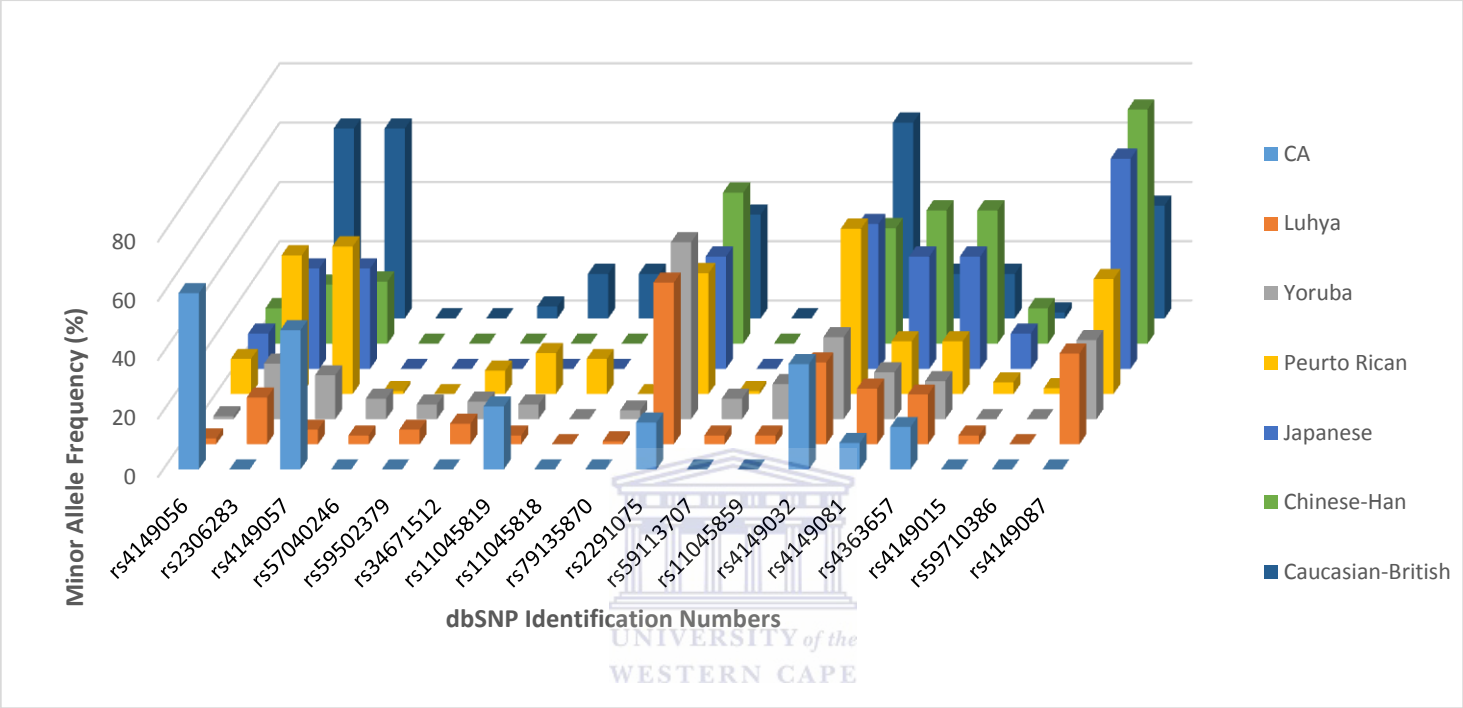


Figure 3.4.1. Allele frequencies of selected *SLCO1B1* SNPs in the CA population compared to other African and world populations.

Table 3.4.3 Haplotype structure defined by 20 SNPs in the *SLCO1B1* gene in the CA population.

Haplotype No.	Haplotype [a]	Frequency %
1	TATCGACGACCCGATGTGAT	10.8
2	CATCGACGACCCGATGTGAT	9.1
3	TACCGACGACCCGATGTGAT	7.9
4	CACCGACGACCCGATGTGAT	7.7
5	CATCGACGACCCGACGTGAT	5.7
6	CATCGACGATCCGATGTGAT	5.3
7	CACCGACGACCCGACATGAT	4.6
8	CACCGACGACCCGACGTGAT	3.9
9	TATCGAAGACCCGATGTGAT	3.6
10	CACCGACGACCCGATGCGAT	2.9
11	TATCGACGACCCGATGCGAT	2.8
12	CACCGAAGACCCGACGTGAT	2.5
13	TACCGACGACCCGACGTGAT	2.5
14	TATCGAAGACCCGACGTGAT	2.5
15	CACCGACGACCCGACACGAT	2.4
16	CATCGAAGACCCGATGTGAT	2.2
17	TACCGACGATCCGATGTGAT	2.1
18	TATCGACGACCCGACGTGAT	1.7
19	CACCGACGATCCGACGTGAT	1.5
20	TACCGAAGACCCGACGTGAT	1.5
21	CATCGAAGATCCGATGTGAT	1.4
22	CACCGAAGACCCGATGTGAT	1.3
23	TACCGAAGACCCGATGTGAT	1.2
24	CACCGACGACCCGACGCGAT	1.1
25	CACCGACGATCCGATGTGAT	1.1
26	CATCGACGACCCGATGCGAT	1.1
27	TATCGAAGATCCGATGTGAT	1.1
28	TATCGACGATCCGACGTGAT	1.1
29	CATCGAAGACCCGATGCGAT	1.0
30	CATCGAAGATCCGACGTGAT	1.0
31	CACCGAAGACCCGACATGAT	0.9

32	TACCGACGACCCGACGCGAT	0.9
33	CATCGACGACCCGACGCGAT	0.6
34	CATCGACGATCCGACGTGAT	0.6
35	CACCGAAGACCCGATACGAT	0.5
36	CACCGACGACCCGATATGAT	0.5
37	CATCGAAGACCCGACGCGAT	0.5
38	CATCGACGATCCGACGCGAT	0.4
39	TACCGAAGATCCGATGTGAT	0.2
40	CACCGAAGACCCGACACGAT	0.1
41	CACCGACGATCCGATGCGAT	0.1
42	CATCGACGATCCGATGCGAT	0.1
43	TACCGAAGATCCGACGTGAT	0.0
	TOTAL	100

a) Haplotype sequences are based on the position of SNPs on chromosome 12.

3.5. Discussion

This study is the first study to report on the allele and genotype frequencies for fourteen nonsynonymous and six intronic SNPs of *SLCO1B1* in the Cape Admixed population of South Africa. A total of 43 haplotypes were identified for the CA population. This study provides important data for ethnic populations of South Africa which could be further investigated within an in vitro, in vivo and clinical setting.

Recently, much attention has been focused on the genetic variation of drug transporters. This is a result of the roles these transporters play in gastrointestinal absorption, biliary and renal elimination and distribution to target sites of their specific substrates (Choi and Song, 2008b). Moreover, emerging pharmacogenomic evidence suggests that membrane transporters are subject to both genotypic and

phenotypic variation (Collins et al., 1998). Additionally, it is also suggested that variation in drug transporters may be the underlying cause of inter-individual variability in pharmacokinetic disposition, efficacy and toxicity of drug transporter substrates (Choi and Song, 2008b).

SLCO1B1 is thought to directly influence the meglitinide class of oral antidiabetic drugs. Repaglinide is a short-acting meglitinide analogue that enhances glucose-stimulated insulin secretion from pancreatic beta cells, thereby reducing blood glucose concentrations (Dornhorst, 2001). Its mechanism of action is binding and closing the ATP-sensitive K^+ channels. Binding and closing the K^+ channel serves to depolarize the beta cell, allowing for the opening of voltage-gated Ca^{++} channels (Hatorp, 2002). This results in the influx of Ca^{++} ions which triggers the release of insulin by exocytosis. However, insulin release is dependent on the beta cell secretory capacity (Hatorp, 2002). The V174A variant has been shown to directly affect repaglinide plasma levels (Kajosaari et al., 2005). Additionally, V174A is associated with a weaker lipid response to statin therapy. In a study by (Rodrigues et al., 2011) it was concluded that this polymorphism causes significant increase in atorvastatin response and may be an important marker for predicting efficacy of lipid-lowering therapy. This is particularly evident when atorvastatin is used in combination with repaglinide. It is suggested that the atorvastatin acts as an inhibitor, thus inhibiting OATP1B1mediated hepatic repaglinide uptake (Chung et al., 2005, Lee et al., 2005, Mwinyi et al., 2008, Niemi et al., 2004, Nishizato et al., 2003).

Furthermore, *SLCO1B1* has been shown to transport methotrexate *in vitro* (Abe et al., 2001, Tirona et al., 2001). Previous studies have confirmed the functional consequences of nonsynonymous polymorphisms in *SLCO1B1* (Kameyama et al.,

2005, Tirona et al., 2001). (Trevino et al., 2009) concluded that for this variant, as well as other less studied SNPs, a lower methotrexate clearance was observed. This is consistent with numerous preceding studies which demonstrated greater plasma exposure to several *SLCO1B1* substrates in those carrying the V174A polymorphism (Ho et al., 2007, Niemi, 2007, Pasanen et al., 2007).

Several pharmacogenetic studies have focused on the potential contribution of *SLCO1B1* genetic variations to statin response. The N130D variant has been associated with elevated activity of OATP1B1 and lower statin concentration in plasma (Mwinyi et al., 2008, Romaine et al., 2010). In addition, the *SLCO1B1**15 haplotype, formed by both N130D and V174A variants, has been associated with decreased activity of the OATP1B1 transporter (Kalliokoski et al., 2010). However, this variant was not observed in the study population, which is curious as it is observed with a high MAF in highly admixed populations, such as the Brazilian population, as well as in homogenous populations such as the British Caucasian population. Furthermore, this may be result of the genetic contributions from Caucasian (Dutch, German and British) and indigenous South African populations present in the study population. Supplementary investigation into the genetic lineage of the study participants and the origin of this variant is suggested.

Studies have determined that he synonymous L191L variant, despite its high prevalence, has no effect on the overall function of the OATP1B1 protein, and additionally, no effect on hepatic expression of *SLCO1B1* (Prasad et al., 2014, Lee et al., 2011). No significant observable effects have been characterised when this SNP exist singly or as part of a haplotype (Lee et al., 2011).

Remarkably, the P155T variant has been shown to enhance the proportional lipid reduction in fluvastatin therapy (Hopewell et al., 2013, Couvert et al., 2008). The observed MAF for the CA population was relatively high at 21.5%. This, in conjunction with the V174A variant, may suggest an increased efficacy of the statin class of lipid-lowering drugs. However, it is suggested that further *in vivo* investigation is required to determine the overall effect of the haplotypes observed within the CA population. Additionally, *in vivo* studies conducted by (Kwara et al., 2014) have suggested that the mechanism for the increased rifampin clearance and lower plasma exposure in subjects with the *SLCO1B1* P155T variant genotype could be due to gain in transporter activity associated with this SNP. They further concluded that males with the P155T variant are at increased risk of lower rifampin plasma exposure (Kwara et al., 2014). The observed MAF in the study population was significantly higher than those it was compared with.

Epidemiological studies often summarize the pattern of arsenic metabolites in urine as proportions of the 3 main inorganic arsenic metabolites, namely; inorganic arsenic (iAs), mono-methylarsonate (MMA), and di-methylarsinate (DMA) (Gribble et al., 2013). A recent study evaluated the association of variants in *SLCO1B1* with arsenic metabolite patterns in urine of participants from the Strong Heart Study, a cohort recruited from rural communities of the Southwestern and Midwestern United States exposed to low-to-moderate arsenic levels in drinking water (Gribble et al., 2013). Their results showed that the minor allele of F199F were significantly associated with variability in %MMA and %DMA accounting for 5% of the residual variance for %DMA arsenic species (Gribble et al., 2013). Interestingly, the observed MAF in the CA population (16%) was considerably lower than all other populations (**Table 3.4.2**).

The *SLCO1B1* rs4149032 polymorphism is associated with low-level rifampin exposure (Chigutsa et al., 2011b). Interestingly, the *SLCO1B1* rs4149032 polymorphism was noted to occur at an uncannily high rate among South Africans. Recent studies have reported lower rifampin concentrations in a high proportion of patients suffering from tuberculosis, consistent with reports from other African populations. Moreover, it has been determined that the lower rifampin concentration observed in African populations, and the study population, may be accounted for by the rs4149032 variant allele (Chigutsa et al., 2011a). The observed MAF was 36% for the CA population, compared to 28% Yoruba (Nigeria) and 66% Caucasian (Britain). Since a large proportion of the CA population are carriers of the rs4149032 polymorphism, it may be suggested that increasing the rifampin dose across the board would be beneficial to not only individuals carrying the variant allele, but also individuals without the variant as higher doses are more likely to be effective and appear to be safe (Chigutsa et al., 2011b).

The intronic SNP rs4149081 in SLCO1B1 was associated with the LDLC response to statins in Chinese patients, with a MAF of 45% (Hu et al., 2012). Furthermore, gastro intestinal toxicity of methotrexate was associated with this SNP, as shown in a study conducted by (Trevino et al., 2009). Although the MAF for this variant was relatively low at 9%.

3.6. Conclusions

Numerous sequence variations have been characterized in the *SLCO1B1* gene, many associated with altered transporter activity *in vitro* and *in vivo*. However, given the understudied genomic diversity harboured within sub-Saharan Admixed and indigenous African populations, the potential exist that novel and rare low frequency

variations could be identified and systematically investigated in the CA population. Admixed ancestry can potentially provide the intrinsic variability needed to unravel intricate gene-environment interactions, which may help explain the population differences in the epidemiology of complex disease. It is thus proposed that investigation of the *SLCO1B1* polymorphisms present in the indigenous African and Admixed populations be initiated, and the effects thereof on statin, repaglinide and methotrexate pharmacokinetics and pharmacodynamics be identified and examined. Moreover, it is suggested that the interaction between these, and other, combinations of drugs also be investigated. This study also lays the foundation for future association studies between *SLCO1B1* variations and treatment outcomes in the CA population. Therefore, **Chapter 4** will prioritize the investigation of promoter variants of the *SLC* genes in the CA participants.

CHAPTER 4

Identification of Genetic Variations within Pharmaco-gene Promoters in the Cape Admixed Population

4.1. Abstract

The solute carrier transporter (SLC) superfamily is a large family of membrane-bound proteins that share 20-25% of sequence homology. Furthermore, it is recognized that members of the SLC family are involved in the facilitated transport of a variety of substances including drugs, environmental toxins, xenobiotics, and endogenous metabolites across plasma membranes. Additionally, it is estimated that genetic factors account for at least 15-30% of variations in drug disposition and responses. Moreover, genetic polymorphisms in drug transporters are increasingly being recognized as a possible mechanism explaining this variation in drug disposition and response. However, to date only a few studies have explored the genetic diversity harboured in the pharmacogenomically relevant *SLC* genes of indigenous and admixed southern African populations. Therefore, the aim of this study was to determine whether the studied *SLC* genes of the CA participants harbours any novel SNPs using direct sequencing of the promoter regions in 96 of the participants. Of the six gene promoters studied, eighteen known variable sites were identified in this study. The number of variants observed in *SLCO1B1*, *SLCO1B3*, and *MATE1* were four, six and eight respectively. The haplotypes inferred for the Cape Admixed population in this study is an important step in filling the gap with regards to genetic information on the pharmacogenomically relevant genes in indigenous southern African populations. The information generated in this study can potentially lay the foundation for future pharmacogenomic study design

and for the identification of association between the rare SNPs and drug response and toxicity.

4.2. Introduction

The solute carrier transporter (SLC) superfamily is a large family of membrane-bound proteins that share 20-25% of sequence homology (Hediger et al., 2004). SLCs are expressed in most tissues. However, these proteins are expressed most abundantly in the liver, kidney, and intestine where they are either located at the basolateral or apical plasma membranes of polarized cells (Wojtal et al., 2009). Today it is acknowledged that members of the SLC family are involved in the facilitated transport of a variety of substances including drugs, environmental toxins, xenobiotics, and endogenous metabolites across plasma membranes (Hediger et al., 2004, Koepsell et al., 2007b). Moreover, these SLC transporters play a critical role in the absorption and excretion of drugs in the kidneys, liver, and intestine, thus, influencing the pharmacodynamic and pharmacokinetic characteristics of these drugs (Meier et al., 2007b).

Previous studies have shown that human SLCs are highly polymorphic in ethnically diverse populations (Sakata et al., 2004, Shu et al., 2003, Kang et al., 2007a). Functional characterization have revealed that several of these SNPs affect the transport function of these genes *in vitro* (Leabman et al., 2002a). Although *in vivo* evidence for the involvement of these SNPs in clinical phenotype is limited, for example, recent studies have shown that homozygous carriers of the hOCT2 variant A270S (rs316019) have a lower renal clearance of metformin compared to those carrying the homozygous wildtype (Song et al., 2008a, Wang et al., 2008a).

However, these pharmacogenomic association studies have primarily been conducted in non-African populations, usually Western European and North American Caucasians, and have focused on genetic variants which are common to these populations (Urban, 2010). The results of these studies are often extrapolated for use and interpretation in other populations. This is in spite of the fact that variant allele frequencies in pharmacogenomic genes can differ significantly between populations and even within populations (Drögemöller et al., 2010, Yen-Revollo et al., 2009). Additionally, population-specific variants exist in non-Caucasians which may more likely be of greater relevance to treatment/study outcomes then those found in Caucasians.

Although it is widely accepted that African populations harbour more genomic diversity than non-African populations, this genetic diversity is however understudied (Frazer et al., 2009, Hardy et al., 2008, Tishkoff et al., 2009). However, in recent years African populations have started to attract research interest, with especially northern and central African countries being increasingly incorporated into studies assessing population structure (Ramsay, 2012, Tishkoff et al., 2009). The Hap-Map and 1000 Genomes projects currently include information on the Luhya and Maasai of Kenya, Yoruba and Esan of Nigeria, Gambian of The Gambia, and the Mende of Sierra Leone. However, the current opinion is that the population genetics of these groups cannot represent the total genomic diversity of the remaining populations in West and East Africa, nor the populations residing in southern Africa (Ikediobi et al., 2011, May et al., 2013). Moreover, the role of this genetic diversity in disease pathogenesis and treatment is currently not fully understood.

CHAPTER 4

The potential importance of gene regulation in disease susceptibility and other inherited phenotypes has been underlined by the observation that the human genome contains fewer protein coding genes than expected (Hoogendoorn et al., 2003).

Promoters are involved in initiating transcription and are therefore among the many important *cis*-acting elements that regulate gene expression that might harbour functionally relevant polymorphisms (Hoogendoorn et al., 2003). However, they differ from most regulatory elements in that their locations are fixed relative to the positions of their respective genes (Hoogendoorn et al., 2003).

Therefore, the aim of this study was to determine whether the *SLC* genes of the CA participants harbours any novel SNPs, using direct sequencing of the promoter regions of these genes in 96 healthy individuals. Secondly, to determine the promoter haplotype structure of the *SLC* genes based on the genetic information acquired by sequencing, and finally, to compare the minor allele frequencies obtained for the Cape Admixed participants to the Hap-Map estimates for other African, American, European and Asian populations.

4.3. Materials and Methods

4.3.1. Subjects

The DNA from 96 of the 130 unrelated healthy CA subjects (As described in **Chapter 2**) was used to screen for novel promoter SNPs by direct sequencing.

4.3.2. DNA extraction and direct sequencing

Genomic DNA samples were collected in the form of buccal swabs and were extracted using a standard salt lysis method as described in Chapter 1 (Leat et al., 2004). The proximal promoter region of the selected *SLC* genes were generated

using self-designed primer sequences. These primers were designed using Primer3 software (www.genome.wi.mit.edu/cgibin/primer/primer3) and obtained from Integrated DNA Technologies (San Diego, California, USA). The PCR reactions were performed in a 50 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qiagen multiplex TopTaq master mix (Qiagen, Courtaboeuf, France) and 0.2 μM of each forward and reverse primer. The PCR conditions included an initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing for 1 minute, and extension at 72°C for 1minute, and then a final extension at 72°C for 10 minutes. The sequences and details of all the primers used in this study are listed in Table **4.4.1**. The fragments were sequenced at Inqaba Biotec (Johannesburg, South Africa).

4.4. Results

4.4.1. Variant Screening

To identify variants, the proximal promoter region of each gene in 96 Cape Admixed participants was screened using direct sequencing. Out of the six gene promoters studied, eighteen known variable sites were identified in this study **(Table 4.2)**. Four variants were observed in *SLCO1B1,* six were detected in *SLCO1B3*, and eight were identified in *MATE1*. No variant SNPs were observed in the *OCT1*, *OCT2*, *OCT3*, and *MATE2-K* promoter regions. Novel variant sites in the six promoter regions were not observed.

Table 4.4.1 PCR primer sequences for promoter region amplicon generation.

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Amplicon length (bp)
SLC22A1	GCCCTACCAAACTGCAAAGC	ACATCTGGAAGGCAACCAAG	818
SLC22A3	GAAGTTCTGATCGCGCTCTG	CTCTCTTTACTCCGCCGCCT	879
SLC47A1	TTGGGAGGTGACGGTTAAGA	CAGGAGCAGCAGAGACTCTC	765
SLC47A2	GTAGCCATGTTTTCACTTTCATAGC	CGGAACCTGAGATACTTTGAGC	698
SLCO1B1	AGGGTCTGTACTTTATTTCAGAAGA	AAACAAGTCCAGTGATGATTAACCA	682
SLCO1B3	ACAGCCATGTGCCTGAGATA	CAAGTCCATCCTTTTTCTGATG	728

Table 4.4.2 Summary of SNPs identified with direct sequencing of the promoter regions of pharmaco-genes in the CA population.

dbSNP ID	Gene	Nucleotide Position Accession number (NC_000006.12)	Position from the translational initiation site or the nearest exon	Nucleotide change and flanking sequence (5′ to 3′)	MAF	HWE
rs73598368	*SLCO1B1*	21130586	712	G>A	0.016	0.876
rs372930030	*SLCO1B1*	21130664	634	T>A	0.010	0.011
rs11835045	*SLCO1B1*	21130885	413	T>C	0.057	0.355
rs71581973	*SLCO1B1*	21131013	285	C>G	0.005	0.003
rs745982712	*SLCO1B3*	20810139	691	A>G	0.005	0.003
rs769039801	*SLCO1B3*	20810268	562	C>T	0.016	0.024
rs770749398	*SLCO1B3*	20810281	549	C>T	0.010	0.011
rs776382139	*SLCO1B3*	20810289	541	T>G	0.010	0.011
rs763010829	*SLCO1B3*	20810424	406	G>A	0.005	0.003
rs60103218	*SLCO1B3*	20810466	364	C>A	0.026	0.069

rs763522289	*MATE1*	1494757	1543	G>A	0.005	0.003
rs115229514	*MATE1*	1494774	1560	C>T	0.005	0.003
rs374132895	*MATE1*	1494782	1568	A>G	0.005	0.003
rs191898031	*MATE1*	1494810	1596	C>T	0.010	0.011
rs7225894	*MATE1*	1494825	1611	C>G	0.005	0.003
rs570369352	*MATE1*	1494838	1624	T>A	0.005	0.003
rs553276075	*MATE1*	1494990	1776	T>A	0.005	0.003
rs555153997	*MATE1*	1495183	1969	C>G	0.005	0.003

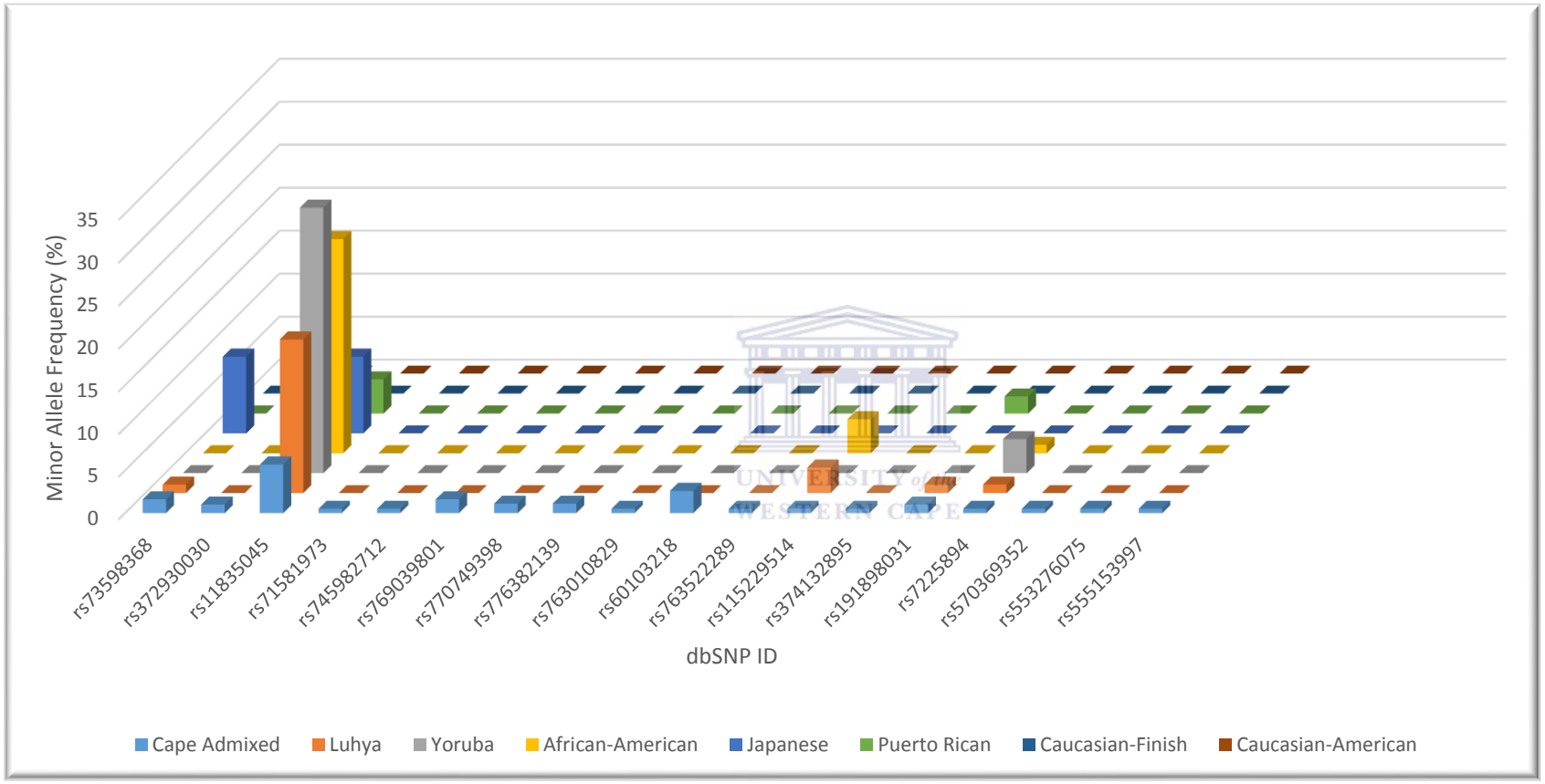


Figure 4.4.1. Minor allele frequencies of selected promoter SNPs in the CA population compared to other African and world populations.

Table 4.4.3 Comparison of MAF of selected promoter SNPs identified by direct sequencing in the CA population to other ethnic groups.

dbSNP ID	Minor Allele	Minor Allele Frequency (%)							
		Cape Admixed[a]	Luhya[b]	Yoruba[b]	African-American[b]	Japanese[b]	Puerto Rican[b]	Caucasian-Finish[b]	Caucasian-American[b]
rs73598368	A	1.6	1.0	0.0	0.0	9.0	0.0	0.0	0.0
rs372930030	A	1.0	*	*	*	*	*	*	*
rs11835045	C	5.7	18.0	31.0	25.0	9.0	4.0	0.0	0.0
rs71581973	G	0.5	*	*	*	*	*	*	*
rs745982712	G	0.5	*	*	*	*	*	*	*
rs769039801	T	1.6	*	*	*	*	*	*	*
rs770749398	T	1.1	*	*	*	*	*	*	*
rs776382139	G	1.1	*	*	*	*	*	*	*
rs763010829	A	0.5	*	*	*	*	*	*	*
rs60103218	A	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
rs763522289	A	0.5	*	*	*	*	*	*	*
rs115229514	T	0.5	3.0	0.0	4.0	0.0	0.0	0.0	0.0
rs374132895	G	0.5	*	*	*	*	*	*	*
rs191898031	T	1.0	1.0	0.0	0.0	0.0	2.0	0.0	0.0
rs7225894	G	0.5	1.0	4.0	1.0	0.0	0.0	0.0	0.0

rs570369352	A	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
rs553276075	A	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
rs555153997	G	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0

a. This study; b. Data from 1000 Genomes; *. No Data

4.4.2. Haplotype Analysis

Haplotypes were calculated using an inferential procedure (Yong and Lin, 2005). This method identified 13 distinct haplotypes across the three gene promoters. The thirteen major haplotypes are listed in **Table 4.4.4**.

4.5. Discussion

This study is the first study to report on the allele and haplotype frequencies for the eighteen selected promoter SNP sites in the Cape Admixed population of South Africa. A total of 13 haplotypes were identified for the CA population. This study provides important data for ethnic populations of South Africa which could be further investigated within an in vitro, in vivo and clinical setting.

Poly-specific solute carrier transporters (*SLC*s) are involved in the sodium-independent electrogenic transport of small organic cations/anions (OCs/OAs) with different molecular structures (Koepsell et al., 2007b). These organic cations include clinically important drugs (metformin, cimetidine, procainamide), endogenous compounds (dopamine, norepinephrine, and toxic substrates (tetraethylammonium, haloperidol-derived pyridinium metabolite, 1methyl4phenylpyridinium) (Gorboulev et al., 1997a). Based on their substrate properties and tissue distributions, human *SLC*-transporters are thought to play important roles in the biliary and renal excretion of their substrates and the distribution of organic cationic drugs in the liver, kidney, heart, and brain (Jonker and Schinkel, 2004).

In a study by (Endo et al., 2012), the association of *SLCO1B1*, *SLCO1B3* and *ABCC2* polymorphisms with the pharmacokinetics of olmesartan was investigated. Their study reports that rs73598368 was observed at 1.4% in the African-American

population and 0% in the Hispanic and Caucasian populations, respectively. However, there was no remarkable difference in any pharmacokinetic parameters of olmesartan between subjects with and without any major haplotypes in the three transporter genes they tested (Endo et al., 2012). This variant was observed at a MAF of 1.6% in the CA population. In the Luhya and Asian populations, it was observed at 1.0% and 9.0%, respectively (**Table 4.4.3**). This may suggest that this variant is specific to African and Asian populations as it is not observed in any Caucasian, European or South American (admixed) populations.

(Kim et al., 2012) sought to determine whether polymorphisms in three drug transporter genes (*ABCB1*, *SLCO1B1* and *ABCC2*) were risk markers for hepatitis induced by the unusual accumulation of antituberculosis drugs (ATDs). Their study revealed a MAF of 7.1% for the rs11835045 variant in the Korean population, compared to 5.7% in the CA population. Furthermore, the 1000genomes project has observed this variant across multiple populations where MAFs may be as high as 31% (**Table 4.4.3**). However, their study suggests that these drug transporters do not play important roles in the pathogenesis of ATD-induced hepatitis in Korean patients, but reccommend that this negative association be reexamined in other ethnic groups as genetic assocciations may be ethnicityspecific (Kim et al., 2010).

Interestingly, no variants (known or novel) were observed for the OCTs and MATE2. However, the lack of amino acid and genetic diversity observed in hOCT1 and *SLC22A1* and is not unique, as it was also observed within hOCT2and *SLC22A2*, and hOCT3 and *SLC22A3* in other populations (Lazar et al., 2003, Lazar et al., 2008). This absence of genetic diversity merely demonstrates the critical role these transporter performs in the elimination of xenobiotics and the maintenance of cellular and organismal homeostasis (Leabman et al., 2003b, Lazar et al., 2003).

CHAPTER 4

Table 4.4.4 Major haplotypes inferred from 18 promoter SNPs identified with direct sequencing.

Haplotype ID	Haplotype	Frequency (%)
SLCO1B1		
Haplotype *1	GTTC	94.3
Haplotype *2	GTCC	4.2
Haplotype *3	ATCC	0.5
Haplotype *4	AACC	0.5
Haplotype*5	AACG	0.5
SLCO1B3		
Haplotype*1	ACCTGC	97.4
Haplotype*2	ACCTGA	1.0
Haplotype*3	ATCTGA	0.5
Haplotype*4	ATTGGA	0.5
Haplotype*5	GTTGAA	0.5
MATE1		
Haplotype *1	GCACCTTC	99.0
Haplotype *2	GCATCTTC	0.5
Haplotype*3	ATGTGAAG	0.5

Therefore, in a study by Jacobs et al (2015) a novel promoter SNP at position -156, which can potentially alter transcription of the *SLC22A2* gene was discovered. Additionally, two promoter SNPs (rs59695691 and rs150063153) that are found only in African population groups were observed. The effect of these African-specific variations on basal promoter regulation has not yet been determined and will be further investigated in **Chapter 5**.

4.6. Conclusions

For the reason that the power to detect rare haplotypes is low, there are likely to be many more functional haplotypes of low frequency than have observed in this study. Like the common variants, the cumulative effects of a large number of rare functional variants can result in common phenotypes. Consequently, it may be concluded that this data offers empirical credibility to the notion that polymorphisms within promoters may be a common source of phenotypic variation and possibly a source of susceptibility to common disease, particularly since our estimate of the

frequency of functional variation is likely to be somewhat conservative. As a consequence, **Chapter 5** will focus on the effect haplotypes, derived from known and novel variants identified in the hOCT2 promoter in the Xhosa population, might have on gene expression.

CHAPTER 5

Cloning of the Polymorphic *SLC22A2* Promoter Region and Development of Promoter Expression Assay

5.1. Abstract

The characterisation of variation in gene expression is complicated by the potentially minute differences associated with alterations in single alleles, as well as by potential variation between individuals arising from environmental or physiological rather than genetic factors. Promoters are involved in transcription initiation and are thus among the multitude of *cis*-acting elements that, by regulating gene expression, might harbour functionally relevant polymorphisms. However, contrary to most regulatory elements, the promoter location is fixed relative to their respective genes. Therefore, promoters are ideal for largescale analysis and functional annotation. Moreover, the frequencies for SNPs in regulatory elements is largely understudied in African, Sub-Saharan, populations. The aim of the present study was firstly, cloning the polymorphic promoter regions representative of 2 of the haplotypes, and secondly to develop a gene expression assay for the polymorphic promoters observed. In this study, we have observed the relative effect of the abovementioned regulatory SNPs on gene expression using a GFP reporter vector. It was demonstrated that a minor difference existed between expression levels of the two representative haplotypes, when compared to the positive control. Nevertheless, it was confirmed that no significant difference existed in relative expression between the two haplotypes. Furthermore, the effect of different tissue types seems to have little effect on promoter regulation. Thus, it may be concluded that our data lends some empirical credibility to the notion that polymorphisms within promoter regions

may be a common source of phenotypic variation, and a likely source of susceptibility to common disease. The capacity to assess the relative mRNA production should be included in future work as it would deliver a more accurate depiction of the inherent regulatory elements in single haplotype promoter expression.

5.2. Introduction

Biomedical research has the vital goal of understanding the genetic basis of human variation. Differences in the level of expression of certain genes account for a major part of the variation within and among other species, as suggested by previous studies in other organisms (Johnson and Porter, 2000, Levine, 2002). The characterisation of variation in gene expression is complicated by the potentially minute differences associated with alterations in single alleles, as well as by potential variation between individuals arising from environmental or physiological rather than genetic factors (Yan et al., 2002).

Attributed to the unpredictably low number of protein coding genes (Peltonen and McKusick, 2001), it has been suggested that a different spectrum of sequence variants may be responsible for the genetic susceptibility to complex diseases (Mackay, 2001, Toma et al., 2002) compared to missense and nonsense mutations (Hoogendoorn et al., 2003). Included in this spectrum are polymorphisms that alter gene expression. These variants are thought to play a significant role in the inter-individual variation in gene expression, attributed to *cis*-acting genetic mechanisms (Hoogendoorn et al., 2003). Since functional polymorphisms in regulatory regions in the human genome cannot yet be reliably distinguished from those with no

consequential effect by their sequence context, it is imperative that the effect these variants may confer be characterised (Hoogendoorn et al., 2003).

Promoters are involved in transcription initiation and are thus among the multitude of *cis*-acting elements that, by regulating gene expression, might harbour functionally relevant polymorphisms (Hoogendoorn et al., 2003).

However, contrary to most regulatory elements, the promoter location is fixed relative to their respective genes. Therefore, promoters are ideal for largescale analysis and functional annotation (Hoogendoorn et al., 2003). Moreover, the frequencies for SNPs in regulatory elements is largely understudied in African, Sub-Saharan, populations. In a previous study by (Jacobs et al., 2015) this issue was addressed, where the main goal was to provide an estimate of the frequency with which functional sequence variation occurs in not only the proximal promoter, but also the coding regions of the *SLC22A2* gene. The results revealed a rare novel SNP (0.52%) (rs572296424) observed in the promoter region in the Xhosa population at position -156 (**Figure 5.2.1)**, relative to the start of the first exon. Furthermore, basal promoter activity is an important determinant of *SLCA22A2* expression *in vivo*, and may influence the transport function of hOCT2, which in turn may affect the uptake, disposition, and elimination of its substrates. A deletion analysis of the hOCT2 proximal promoter region by Asaka *et al.* (2007) suggested that the region spanning -91 to -58 base pairs (bp) was essential for basal transcriptional activity of the *SLC22A2* gene (Asaka et al., 2007). This region lacks a canonical TATA-box, but does contain a CCAAT box and an E-box. The electrophoretic mobility assays revealed that the *SLC22A2* E-box binds upstream stimulating factor 1 (USF1) which functions as a basal transcriptional regulator of the gene. Jacobs et al (2015) also observed a common SNP (1.6%) (rs150063153) at position -95 relative to the start of

the first exon. Given the proximity of this SNP to the above-mentioned regulatory region, DNA polymerases, USFs and enhancer element may suffer altered binding efficiencies. For these reasons, the rare novel variant (rs572296424) at position -156 (-156 Haplotype) and more common known SNP variant (rs150063153) at position -95 (-95 Haplotype) will serve as the areas of interest for the purpose of this study.

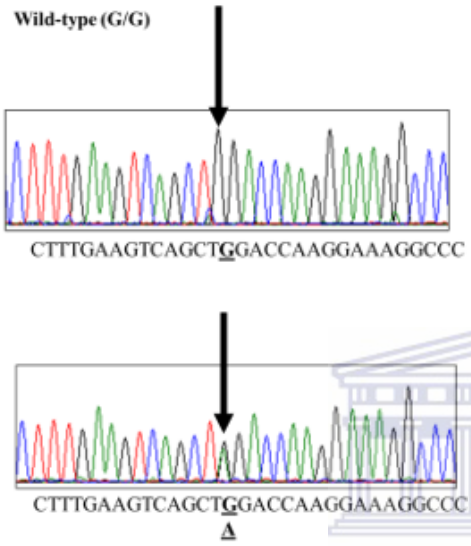


Figure 5.2.1. Electropherograms of novel SNPs observed in the proximal promoter region of the *SLC22A2* gene. MBPG_OCT2001 (wildtype -156 G/G and variant -156 G/A) a novel SNP detected in the proximal promoter region by (Jacobs et al, 2015).

The *SLC22A2* gene, located on chromosome 6q26, encodes the hOCT2 solute carrier transporter (Gorboulev et al., 1997b, Koepsell et al., 2007a, Koehler et al., 1997b). Clinical studies, *in vivo* animal experiments, and meta-analyses have demonstrated that variations in the level of expression of *SLC22A2* may be accountable for the observed inter-individual variation in pharmacokinetics (Jacobs et al., 2015). Furthermore, genetic polymorphisms in *SLC22A2* have been implicated in the altered function of hOCT2, leading to a divergence in the disposition and response to substrate drugs, such as metformin (Jacobs et al., 2015). Thus the aim of the present

study was firstly, cloning the polymorphic promoter regions representative of two of the haplotypes generated by (Jacobs et al., 2015), and secondly to develop a gene expression assay for the polymorphic promoters observed by (Jacobs et al., 2015).

5.3. Materials and Methods

5.3.1. Promoter selection and variant identification

The hOCT2 promoters used, representative of the haplotype associated with the novel SNPs, were identified by (Jacobs et al., 2015).

5.3.2. Primer Design

Primers, **Table 5.5.1**, were designed using Primer3 software (www.genome.wi.mit.edu/cgibin/primer/primer3) and obtained from Integrated DNA Technologies (San Diego, California, USA).

5.3.3. Cloning Reaction

DNA from the samples used by (Jacobs et al., 2015) was amplified using TopTaq (Qiagen, Courtaboeuf, France) according to manufacturer's protocols. TopTaq contains a high-fidelity DNA polymerase to minimize mis-incorporation of nucleotides. The PCR reactions were performed in a 50 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qiagen multiplex TopTaq master mix and 0.2 μM of each forward and reverse primer. The PCR conditions included an initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing for 1 minute, and extension at 72°C for 1minute, and then a final extension at 72°C for 10 minutes. The sequences and details of all the primers used in this study are listed in Table **4.4.1**. PCR products were modified to have T/A overhangs and were ligated into a pGLOW vector (Invitrogen, California, USA),

which contains a green fluorescent protein gene (GFP), and cloned into Top10 super-competent cells by T/A cloning as outlined by the manufacturer (Invitrogen, California, USA). Plasmid DNA was purified using standard protocols and sequenced using Big Dye terminator v3.1 (ThermoFisher Scientific, California, USA) in both directions to confirm the haplotype present.

5.3.4. Cell Culture

The ability of each sequence to promote transcription of the GFP gene was tested transiently in human cell lines HEK293 (Human embryonic kidney cells) (Cellonex, Randburg, South Africa) and LnCAP (human prostate adenocarcinoma cells) (ATCC, Manassas, USA). Cell lines were transfected using ScreenFect A (InCella, Eggenstein-Leopoldshafen, Germany) in a 6well format (two replicates per clone), and cultured according to standard protocol at 37°C and 5% CO_2. GFP expression was confirmed by fluorescent microscopy.

5.3.5. Flow Cytometry

Quantification of GFP expression was carried out using the BD Accuri C6 flow cytometer. Cell suspensions of each cell line were passed through a 488 nm laser for excitation, and fluorescence emissions were collected at 530 nm (+/ 15 nm) for GFP. Plots were gated against side scatter to exclude cell debris. Samples were analysed using fluidics set to Slow (core size = 16µm, flow rate = 34µl/min), and at least 50,000 events were collected per sample.

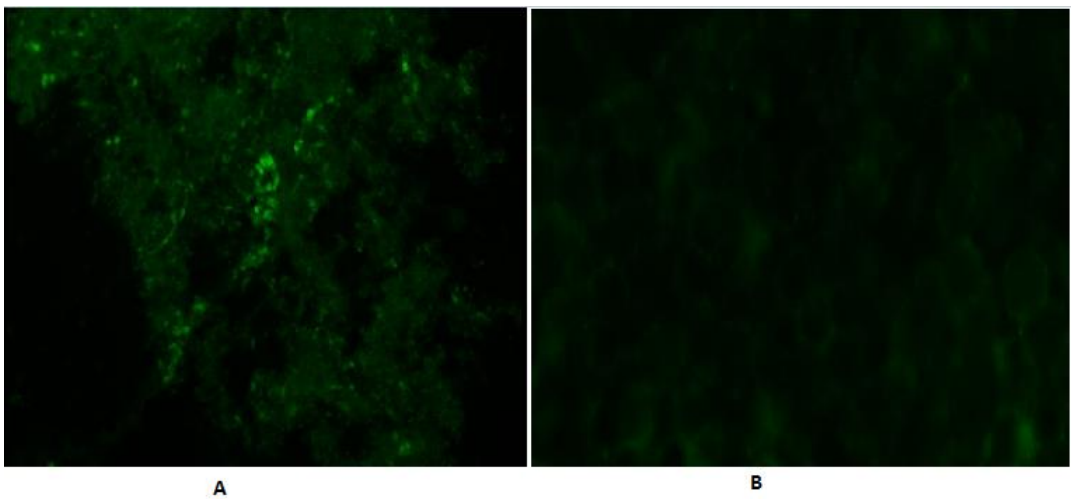
Table 5.3.1 PCR primer sequences for cloning the promoter region in-frame.

Primer sequence (5'3')	Amplicon length (bp)

F Primer	GGGGTACCATCCTAAGGCTCACGGCCAAC	1025
R Primer	CGACGCGTTCACAGCCCAGTAATCTTCCC	

5.4. Results

All cloned haplotypes were examined for their ability to drive transcription of the GFP reporter gene in two human cell lines, HEK293 and LNCaP, using a 6well culture format. For the measurement of fluorescence using flow cytometry, gating was determined by measuring untransfected cells. This allowed for the size and location, on a scatter plot, to be isolated and excluded from the measurement of representative haplotype fluorescence. Cell debris, untransfected cells and crystals in the tissue culture media, and fluorescence of the media itself, was observed to occur between the ranges of 10^1 and 10^3. Correspondingly, fluorescent emission was observed to occur in the ranges 10^3 to 10^6. This range, according to the manufacturers specifications, is the ideal area for GFP detection. Therefore, all samples were gated for this range. Furthermore, GFP expressing cell counts in the LNCaP cell line were noted to be lower than those in the HEK293 cell line. This is expected as *SLC22A2* is primarily expressed by the kidneys.



A B

Figure 5.4.1. Confirmation of GFP expression by fluorescent microscopy. A) HEK293 cells expressing GFP with cloned -95 haplotype. B) LNCaP cell expressing GFP with -95 haplotype.

5.5. Discussion

The principle of this study was to determine whether SNPs in the proximal promoter region of the *SLC22A2* gene would have any adverse effect on expression. It is well documented that variations near or within consensus sequences, such as the TATA-box or GC-box and other *cis*-elements, are responsible for variations in expression levels of their associated genes (Ogasawara et al., 2008).

In this study we examined two distinct regulatory SNPs (rSNPs), a novel rare variant identified in the Xhosa population at -156bp and a known variant at -95bp. It is expected that the proximity of these rSNPs to regulatory sites (*cis*-elements) could alter the binding efficiency of transcription factors, polymerases and enhancers (Saji et al., 2008). Furthermore, clinical studies, *in vivo* animal experiments, and meta-analyses have demonstrated that variations in the level of expression of *SLC22A2* may be accountable for the observed inter-individual variation in pharmacokinetics (Jacobs et al., 2015, Sakurai et al., 2004).

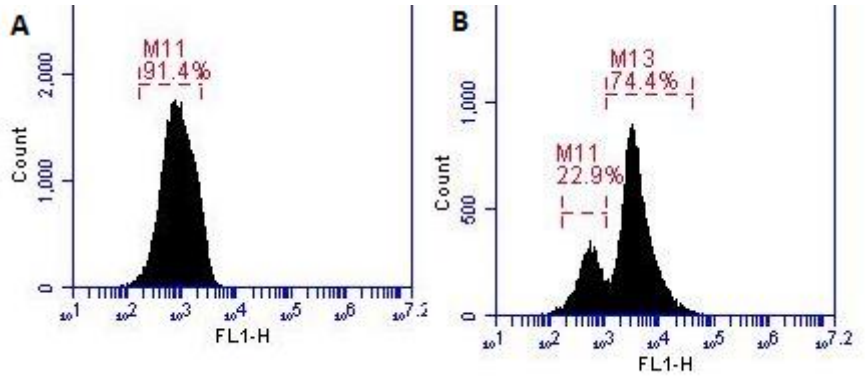


Figure 5.5.1 – Relative fluorescence detected by flow cytometry. A) Untransfected HEK293 cells. B) HEK 293 cells transfected with the positive control, expressing GFP. Where M11 indicates cell debris and M13 indicates fluorescence.

In this study, we have observed the relative effect of the abovementioned rSNPs on gene expression using a GFP reporter vector. The results obtained within the present work are a double-edged.

Initially, it was demonstrated that a minor difference existed between expression levels of the two representative haplotypes, when compared to the positive control (**Figure 5.5.2**). This may be due to fluctuations in the number of surviving cells which were able to express GFP. However, it was also confirmed that no significant difference existed in relative expression between the two haplotypes. Furthermore, a second method of fluorescent measurement, luminometric, was used to corroborate these findings (**Table 5.5.1**). The GloMax Multi detection System (Promega Corporation, Madison, USA), set to excitation at 525nm and emission detection at 580-640nm, was used.

Table 5.5.1 – Mean fluorescence measured in 23 replicates on green filter using a luminometer.

Untransfected cells	+ve control	⁻95 Haplotype	⁻156 Haplotype
0	1	0,96	0,93
0	1	0,94	0,94
0	1	0,92	0,92
0	1	0,95	0,93
0	1	0,97	0,94
0	1	1,50	1,35
0	1	1,23	1,28
0	1	1,03	1,11
0	1	1,31	1,22
0	1	0,77	0,78
0	1	0,74	0,73
0	1	0,72	0,72
0	1	0,80	0,78
0	1	0,97	0,92
0	1	0,97	0,94
0	1	0,93	0,93

0	1	0,95	0,90
0	1	0,90	0,92
0	1	0,92	0,93
0	1	0,93	0,86
0	1	0,96	0,86
0	1	0,98	0,90
0	1	0,89	0,94

Standard error: -95 haplotype = 0.04, -156 haplotype = 0.03

Once more the minor difference between the positive control and representative haplotypes was observed. In addition, the same lack of significant difference in fluorescence was observed between the two representative haplotypes. The average fluorescence detected between the single haplotypes existed between 0.95 – 0.97, after correcting for fluctuations is cell density, with a 95% CI of 0,8908 to 1,0429 for the -95 haplotype and 0,8775 to 1,0134 for -156 haplotype.

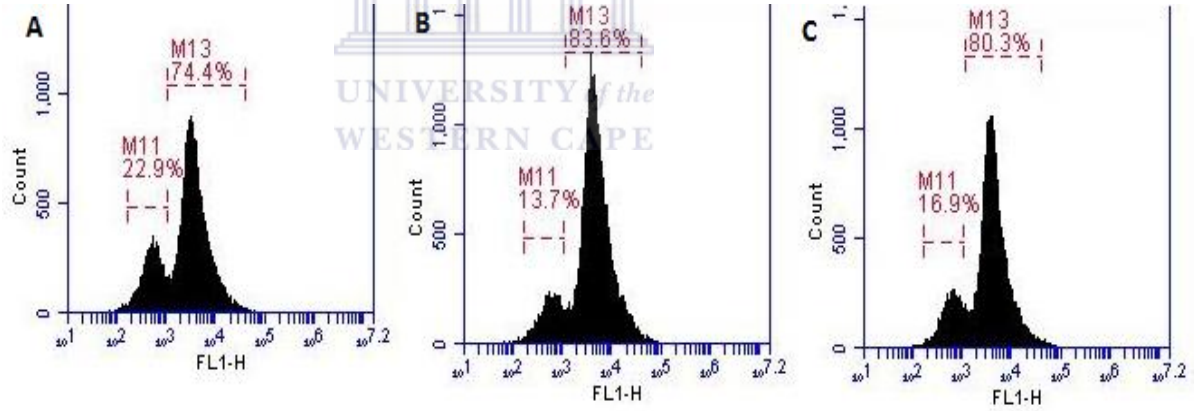


Figure 5.5.2 – Fluorescent measurement of transfected HEK293 cells, where M11 indicates cell debris and untransfected cells, and M13 is indicative of fluorescence. A) positive control expressing GFP. B) 95 Haplotype, transfected cells expressing GFP. C) 156 Haplotype, transfected cells expressing GFP.

Furthermore, the effect of different tissue types seems to have little effect on promoter regulation (**Figure 5.5.3)**. This may be due to qualitative differences in the

response of the cell lines to individual promoter haplotypes and demonstrates the importance of using multiple cell lines in these analyses (Hoogendoorn et al., 2003).

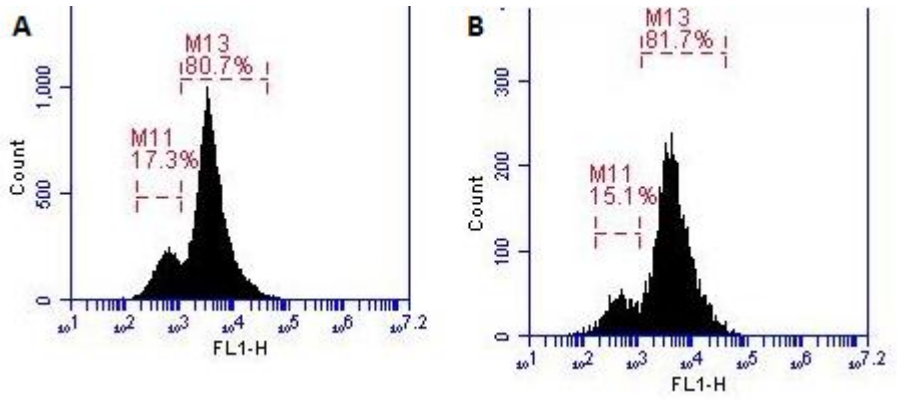


Figure 5.5.3 - Fluorescent measurement of transfected LNCaP cells, where M11 indicates cell debris and M13 is indicative of fluorescence. A) -95 Haplotype, transfected cells expressing GFP. B) -156 Haplotype, transfected cells expressing GFP.

However, it must be noted that the results discussed above are measured at P>0.05. At P>0.01 the significance of the result increases theoretically, as the scale of measurement is smaller and can resolve minor differences more effectively. Nevertheless, since these variations have only been observed in understudied African populations thus far, the effect they may produce has yet to be determined. Thorough supplementary investigation will be required to elucidate the phenotypic effects.

It should also be noted that a number of limitations exist within our study. Firstly, estimates of the occurrence of functional variations in regulatory regions are poor. Effects of polymorphisms in the proximal promoter region, studied herein, have not been measured under dynamic states, such as during development or in response to hormonal changes. Secondly, the relative amount of mRNA produced had not been determined. However, the magnitude of mRNA changes *in vivo* is largely unknown

as regulation of gene expression in living tissue, in their natural genomic frameworks, is likely to be more sophisticated than a reporter assay. Correspondingly, it is possible that changes in mRNA may not result in changes in protein abundance or activity as a result of translational and posttranslational regulatory process (Hoogendoorn et al., 2003). It may be suggested that the cumulative effects of a large number of rare, or novel, functional regulatory variants may result in common phenotypes. Moreover, single rSNP haplotypes are tremendously rare, and thus, the cumulative effect of observed SNPs should be extensively studied in order to make accurate predictions promoter activity, and ultimately drug response.

5.6. Conclusions

We may, therefore, conclude that our data lends some empirical credibility to the notion that polymorphisms within promoter regions may be a common source of phenotypic variation, and a likely source of susceptibility to common disease. It should also be noted that this is a descriptive study with the intention of developing and optimizing methods for future expression, and more importantly, drug transport assays. The capacity to assess the relative mRNA production should be included in future work as it would deliver a more accurate depiction of the inherent regulatory elements in single haplotype promoter expression.

CHAPTER 6

Summary and Future Perspectives

It is well documented that genetic variations in drug metabolizing enzymes underlie the inter-individual differences in drug response. A great deal of inter-individual variability exists in drug disposition, therapeutic efficacy, and adverse drug reactions. Although numerous factors can contribute to this variation, it is estimated that genetic factors account for between 15-30% of inter-individual variations in drug disposition, efficacy and adverse responses, and for certain drugs this estimate can even be as high as 95%. However, polymorphisms in solute carrier transporters have been recognized as a contributing factor to inter-individual variation in drug response and being involved in the toxicity of drug treatment or predisposition to adverse drug reactions. These polymorphisms have attracted interest as SLCs are widely distributed in the epithelial membrane of the liver, kidney, and intestine and play an important role in the gastrointestinal absorption, biliary and renal uptake and excretion, and distribution to target sites of their substrate drugs.

Membrane transporters of the SLC superfamily, particularly OCTs and OATs, play a critical role in maintaining organismal and cellular homeostasis. They perform this important function by being involved in the absorption of nutrients essential for cellular metabolism and the elimination of metabolic waste products and toxic xenobiotics. Approximately 40% of therapeutic drugs are organic cations or weak bases at physiological pH and are substrates of organic cation transporters and multidrug and toxin extrusion transporters. The transport of organic cations is mediated by OCTs in an electrogenic manner, independent of a sodium-gradient, and by MATEs through an oppositely directed proton gradient. Examples of clinically

important drugs transported by OCTs and MATEs include the antidiabetic drugs metformin and phenformin, the antineoplastic drugs cisplatin and oxaliplatin, the anti-HIV drugs lamivudine and zalcitabine, and the histamine receptor antagonist cimetidine.

Moreover, the current data suggest that these transporters also play an important part *in vivo* drug disposition, therapeutic efficacy, and adverse drug reactions. Genetic polymorphisms in *SLC22A1-3* and *SLCO1B1* have been associated with reduced transport and efficacy of clinically important drugs such as, for example, the biguanide antidiabetic metformin and the HMG-CoA reductase inhibitor simvastatin. However, these studies with reduced-function variants have primarily focused on genetic polymorphisms that are prevalent within Caucasian and Asian populations. This is in spite of numerous studies which have shown that the genomic diversity found within African populations is greater than the genetic variation found within other populations. However, this genomic diversity, especially in the southern African context, is currently relatively understudied. Given the enormous health burden that sub-Saharan Africa, and for that matter South Africa, faces, this lack of local genetic information with robust allele frequency distribution currently serves as a significant hurdle to designing biomedical research, and may have important medical implications.

Our investigation into the allele and genotype frequency distributions of previously reported nonsynonymous SNPs revealed that *SLC22A1* reduced-function variants found in Caucasians (R61C, C88R, S189L, G401S, and M440I) and Asians (P283L and R287G) were not only absent in the Cape Admixed subjects genotyped in this study, but are also absent from other African populations that are included in the

Hap-Map and 1000 Genomes projects. Given that these variants have been implicated in reduced transport of the antidiabetic metformin, screening for them in the Cape Admixed and African populations still remains clinically relevant.

The high frequency, reduced-function nonsynonymous variants which are common amongst populations such as P341L in *SLC22A1* and A270S in *SLC22A2* have different frequency distributions for African populations when compared to other populations. For example, the average reported MAF for P341L is higher for Asians (14.6%) and Africans (8.5%) compared to that observed for American Caucasians (<1.0%). Conversely, the average reported MAF for A270S is higher for Africans (15.9%) than that recorded for Asians (14.7%), European-Caucasians (5.9%) and American-Caucasians (8.8%).

Furthermore, ethnic-specific nonsynonymous genetic variants that have been observed at allele frequencies >1% within the Xhosa and other African populations, such as S14F, G220V, and V519F (*SLC22A1*) and R176H (*SLC22A2*) have to date not been reported for Asian or Caucasian populations or are very rare in these populations with MAF of <5%. Additionally, the *in vitro* and *in vivo* consequences of these variants have not yet been established. The effect *in vivo* of these Africa-specific nonsynonymous variants on drug disposition, efficacy, and adverse reactions in African patients has not yet been determined, and requires further investigation.

The M420del deletion variant of hOCT1 had been implicated in the reduced transport and efficacy of the antidiabetic drug metformin. In addition, this variant is also associated with increased failure rate of imatinib, an antineoplastic TKI, treatment in CML patients. Although the M420del variant was not observed in the

current study, screening for it in the Cape Admixed and indigenous African populations still remain a priority.

Genotyping of the *SLC22A3* gene, which encode hOCT3, revealed that none of the nonsynonymous SNPs assayed were present in the Cape Admixed subjects screened. However, these polymorphisms are rare variants that occur at very low frequency in a population and therefore the results are consistent with studies conducted in other populations. This lack of protein sequence and mutational variability was ascribed to selective pressures that act at the *SLC22A3* loci and pointed at the important physiological roles the hOCT3 transporter perform *in vivo*. Furthermore, the SNPs typed in this study were previously identified in other populations and therefore we cannot rule out the presence of novel SNPs in the *SLC22A3* gene of Cape Admixed individuals.

Similar to common variants, the cumulative effects of a large number of rare functional variants can result in common phenotypes. As a result, it may be concluded that the data generated herein offers empirical credibility to the notion that polymorphisms within promoters may be a common source of phenotypic variation and possibly a source of susceptibility to common disease, particularly since our estimate of the frequency of functional variation is likely to be somewhat conservative.

In the current study, we have examined the effect of a novel SNP in the *SLC22A2* gene of the Xhosa population in the 5′-untrnaslated region -156 bp from the initiation codon. Our data lends some empirical credibility to the notion that polymorphisms within promoter regions may be a common source of phenotypic variation, and a likely source of susceptibility to common disease. However, since this is a

descriptive study with the intention of developing and optimizing methods for future expression, and more importantly, drug transport assays, the capacity to assess the relative mRNA production should be included in future work. This would deliver a more accurate depiction of the inherent regulatory elements in single haplotype promoter expression.

The sample size of this study compared well to that of the Hap-Map and 1000 Genomes Projects, which have more than demonstrated their value as reference panels for specific populations, but a larger sample size and complete individual gene/genome sequences would give a more accurate account of the full spectrum of genetic diversity within the Cape Admixed population. However, sequencing whole genomes/exomes remains an expensive exercise.

To our knowledge, this is the first study that investigated the allele and genotype frequency distributions of SNPs in the *SLC* genes of the Cape Admixed population. This study also reports the observed haplotypes in the investigated population. It has also been shown that reduced-function nonsynonymous SNPs in the *SLC* genes found in Caucasian and Asian populations are absent from the Cape Admixed population. We have shown that, although MAF observed for the Cape Admixed population is largely similar to other African populations, differences exist that may translate into differences in organic cationic drug transport between these ethnic groups. These variations may translate into differences in the transport and efficacy of organic cationic drugs commonly used for the treatment of diseases prevalent in Africa. However, it should be noted that this was only a descriptive study and that no associations are made between any diseases or treatment outcomes. Although, it is well known that individual variation in drug response can be attributed to specific

genetic variants. Moreover, it is believed that the incorporation of haplotypes in pharmacogenomic studies will provide a more complete picture of loci that are relevant in the practice of "genetic medicine" both at an individual or population level. This study contributes towards filling the gap that exists with regards to genetic information about important variations in organic cation transporter genes, such as *SLC22A1-3*, for the indigenous populations of South Africa given that organic cationic drugs are used in the treatment of diseases such as type-2 diabetes mellitus, various cancers, and HIV, these variants may impact profoundly on healthcare provided over the African continent. Therefore, given the aforementioned reasons studies such as this is valuable in the generation of useful pharmacogenomic information specific for Admixed populations.

These systems have since been routinely used to genotype additional indigenous and admixed populations. The variants included deleterious SNPs which have been implicated in altered pharmacokinetics and pharmacodynamics of the widely-prescribed antidiabetic drug metformin and as a predictor of treatment outcome for the antineoplastic imatinib. Firstly, these genotyping systems can now potentially be used to screen routinely for these reduced-function variants in responders and non-responders to metformin therapy. Secondly, it can also be used to determine whether CML patients carry the M420del allele which is a predictor of imatinib treatment outcome. Thirdly, the genotyping systems for *SLC22A1*, *SLC22A2* and *SLCO1B1* can potentially be used to determine whether certain individuals have a genetic predisposition for drug-drug interactions. Lastly, the genotyping systems developed for the screening of *SLC22A3* SNPs can be used to screen patients with psychiatric disorders in order to determine whether they carry any of the variant alleles included in these systems.

CHAPTER 6

Future studies require a more comprehensive sequencing of the *SLC22A1-3* and *SLCO1B1-3* genes and to expand this to more individuals or, where such sequencing information becomes available from other largescale sequencing projects, use it to determine correlation between genotypes and clinical phenotypes. Furthermore, the effect of the newly identified and the other African-specific promoter SNPs on the expression on *SLC22A2* and on hOCT2 transport function needs to be further assessed in future work. However, it must be noted that these results were observed at 95% confidence interval, and that a 99% confidence interval the significance may increase theoretically. In addition, the cumulative effects of observed SNPs/rSNPs haplotypes on transport kinetics, protein turnover, and plasma membrane localization of the SLC's also requires further investigation in the Cape Admixed and other indigenous African populations, and should also be prioritized in future studies. Therefore, further translational work is also suggested to develop associations between the SLC superfamily and drug response in a clinical setting. Optimization of choice, dose, and method of treatment, need to be developed and prioritised in future studies, particularly in understudied sub-Saharan African populations.

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