PHARMACOGENOMICS OF SOLUTE CARRIER TRANSPORTER GENES IN THE XHOSA POPULATION

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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 pharmacogenetically relevant *SLC22A1-3* and *SLC47A1* genes in the indigenous African population of South Africa. Secondly, to determine whether allele and genotype frequencies for these SNP were different from that reported for other African, Caucasian, and Asian populations. Thirdly, to infer haplotypes from the genetic information which can potentially be used in future to design and interpret results of pharmacogenetics association studies involving these genes and their substrate drugs. Finally, to determine whether the Xhosa population harbour novel SNPs in the *SLC22A2* gene, that encodes the kidney-specific hOCT2. SNaPshot™ multiplex single base minisequencing systems were developed and optimized for each of *SLC22A1*, *SLC22A2*, *SLC22A3*, and *SLC47A1* covering the previously identified 78 SNPs. These systems were then used to genotype the alleles of 148 healthy Xhosa subjects residing in Cape Town, South Africa. In addition, the proximal promoter region and all 11 exons and flanking regions of the *SLC22A2* gene of 96 of the participants were screened for novel SNPs by direct sequencing. The Xhosa subjects investigated lacked heterozygosity and were monomorphic for 91% of the SNPs screened. None of the *SLC22A3* and *SLC47A1* SNPs investigated was observed in this study.

Sequencing of the *SLC22A2* gene revealed 28 SNPs, including seven novel polymorphic sites, in the 96 Xhosa subjects that were screened. The minor allele frequencies of the seven previously identified variant SNPs observed in this study were different compared to that observed for American and European Caucasian, and Asian populations. Moreover, the allele frequencies for these SNPs differed amongst African populations themselves. Eight and seven haplotypes were inferred for *SLC22A1* and *SLC22A2*, respectively, for the Xhosa population from the information gathered with SNaPshot™ genotyping. This study highlights the fact that African populations do not have the same allele frequencies for SNPs in pharmacogenetically relevant genes. Furthermore, the Xhosa and other African populations do not share all reduced-function variants of the *SLC22A1-3* and *SLC47A1* genes with Caucasian and Asian populations. Moreover, this study has demonstrated that the Xhosa population harbours novel and rare genetic polymorphisms in the key pharmacogene *SLC22A2*. This study lays the foundation for the design and interpretation of future pharmacogenetic association studies between the variant alleles of the *SLC22A1-3* and *SLC47A1* genes in the Xhosa population and drug disposition and efficacy.

DECLARATION

I declare that *'Pharmacogenomics of solute carrier transporters genes in the Xhosa population'* 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.

CLIFFORD WINSTON JACOBS NOVEMBER 2014

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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

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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, 2008).

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).

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 sodium-gradient, and by MATEs through an oppositely directed proton gradient (Koepsell *et al.*, 2007, 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.*, 2007). A number of these variants have been associated with reduced effect of therapeutic drugs, example the anti-diabetic metformin and the anti-neoplastic

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.*, 2003).

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 polymorphsisms (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, 2005). 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 pharmacogenetics (Frazer *et al.*, 2009).

1.3. Pharmacogenetics/Pharmacogenomics and Individualized/Personalized Drug Therapy

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

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 population groups (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). 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 prophalaxis, 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 internationally 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.

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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 anemia, Tay-Sachs, and cystic fibrosis for example, differ between populations (Via *et al.*, 2009). 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.*, 2009). 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, 2008).

The accumulated data reveal that allele, genotype and haplotype frequency of polymorphisms of pharmacological relevant genes (pharmacogenes) may differ significantly among populations categorized by race, ethnicity, or continental origin (Suarez-Kurtz). 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 inter-ethnic 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, 2008).

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, 2008, Giacomini *et al.*, 2010). It is estimated that genetic factors generally account for 15-30% of inter-individual variation in drug disposition and response, and for certain drugs genetic factors can account for up to 95% of the inter-individual variation in drug disposition and effects (Avery *et al.*, 2009, Choi and Song, 2008).

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.*, 2003, Yan 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.*, 2003). 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, 2008, 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.*, 2003, Yan Shu *et al.*, 2007).

During the last decade, a greater focus has been given to the 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 pharmacogenetic 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 20-25% 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 trans-membrane proteins which typically have a predicted membrane topology that consists of 12 α-helical 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.*, 2007). 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.*, 2007). 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.*, 2007).

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.*, 2007). 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.*, 1997). 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.*, 2007). Moreover, there are several members of the SLC family for which the substrate specificity and/or function have not been elucidated yet.

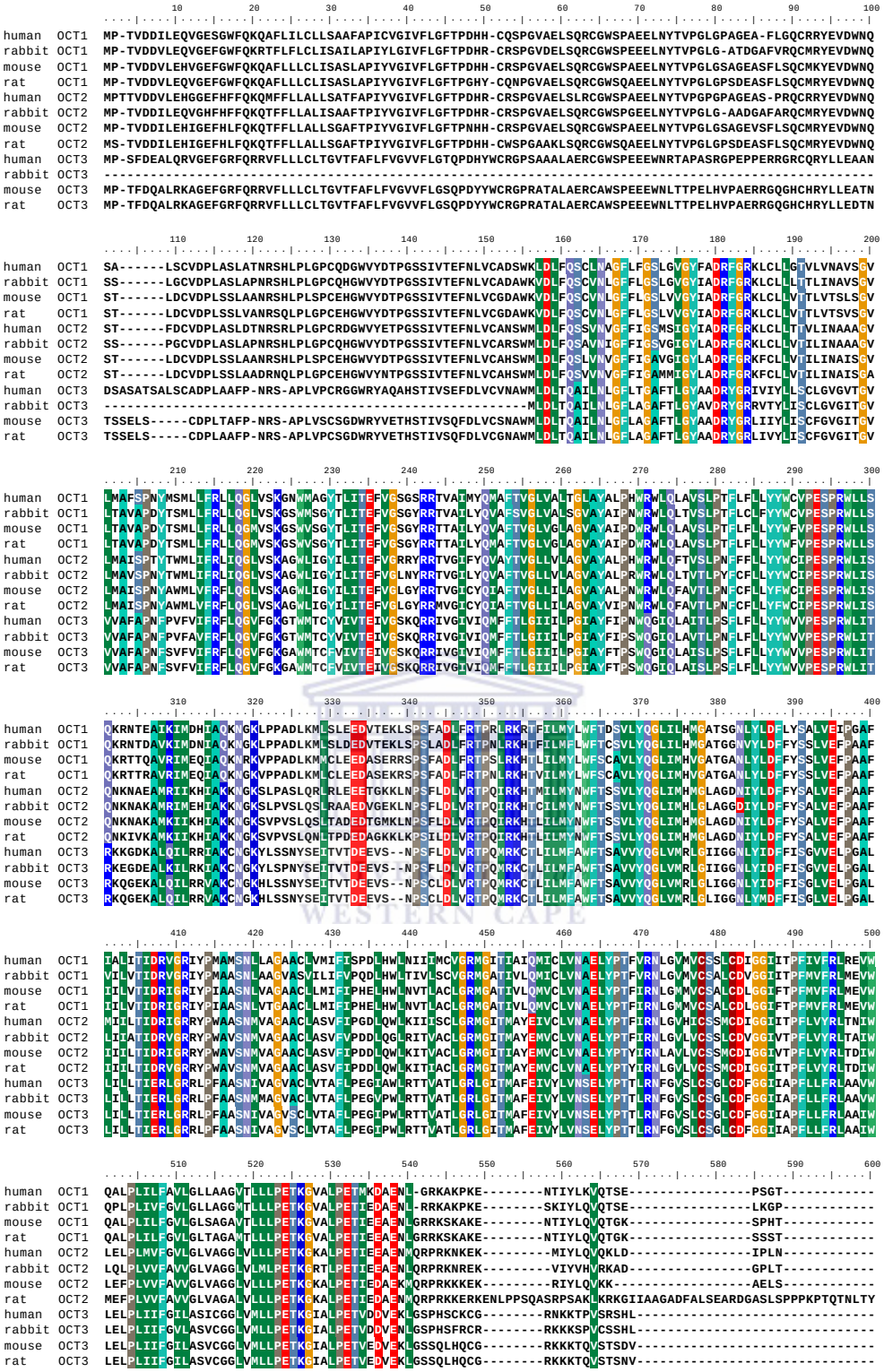

Figure 1.1 - Multiple protein sequence alignment of OCTs from four animal species showing a high degree of evolutionary conservation.

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 α-helices (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.*, 2007).

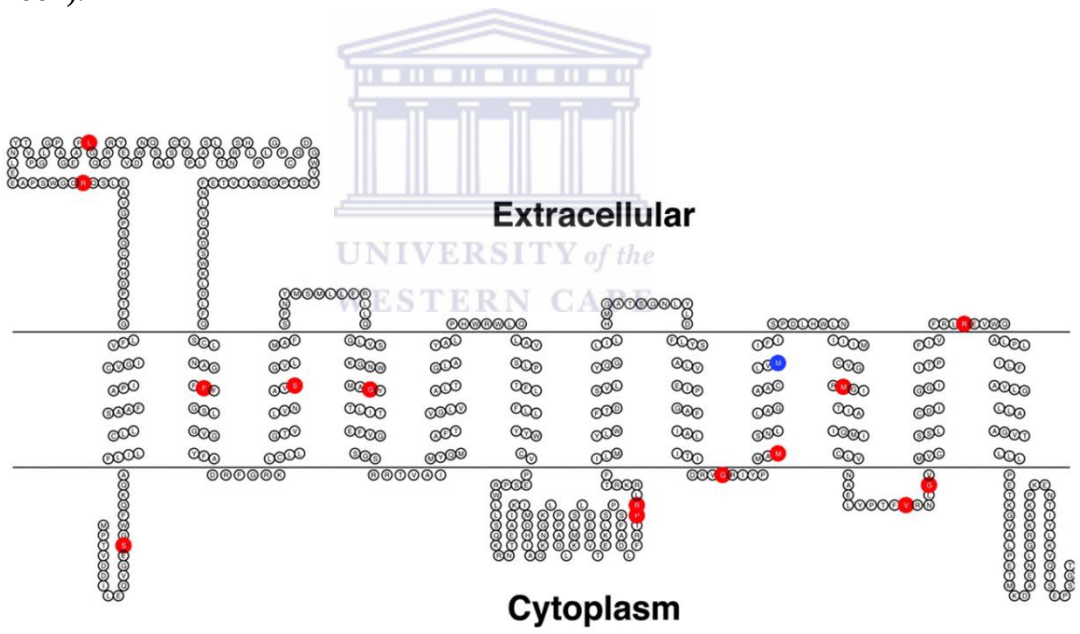

Figure 1.2 - Predicted membrane topology of OCTs as represented by human OCT1 (Shu *et al.*, 2003).

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 **(Tables 1.1-1.3 and Figures 1.3 & 1.4)** (Jonker and Schinkel, 2004, Koepsell *et al.*, 2007). There is extensive overlap of substrate and inhibitor specificities among hOCT1-3 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 **(Table 1.1)** 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 **(Table 1.2 and Figure 1.3)** such as dopamine, epinephrine, norepinephrine, and histamine (Klaassen and Aleksunes, 2010).

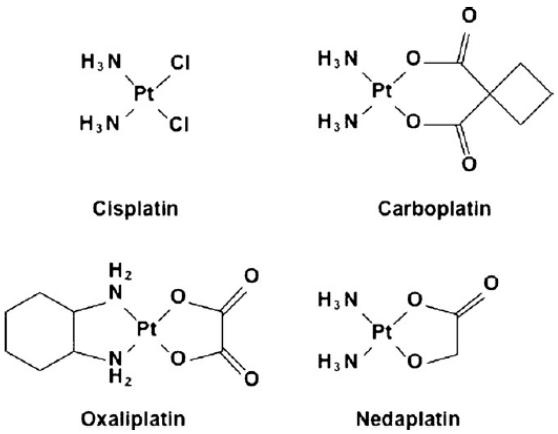

Figure 1.3 - Chemical structures of 4 platinum agents which are substrates of hOCTs and hMATEs (Yokoo *et al.*, 2007).

Table 1.1 - Selected drugs transported by hOCTs and hMATEs.

Drug Category	Typical Drug	Pharmacological Action	References
Anthraquinones	Mitoxantrone	Antineoplastic	(Koepsell *et al.*, 2007)
Aromatic diamidines	Furamidine	Anti-parasitic	(Ming *et al.*, 2009)
Biguanides	Metformin	Antidiabetic	(Shikata *et al.*, 2007, Takane *et al.*, 2008)
Camptothecin analogs	Irinotecan	Antineoplastic	(Gupta *et al.*, 2012)
Cationic steroids	Rocuronium	Neuromuscular blocking	(Van Montfoort *et al.*, 2001)
Dopamine agonists	Pramipexole	Anti-Parkinsonian	(Diao *et al.*, 2010)
Dopamine antagonists	Sulpiride	Antidepressant	(Koepsell *et al.*, 2007)

Table 1.1 Continued - Selected drugs transported by hOCTs and hMATEs.

Fluoroquinolones	Ciprofloxacin	Antimicrobials	(Ciarimboli *et al.*, 2013)
Histamine Antagonists	Cimetidine	Antigastric ulcers	(Tahara *et al.*, 2005, Zhang *et al.*, 1998)
NMDA receptor antagonists	Memantine	Anti-Parkinsonian	(Amphoux *et al.*, 2006)
Nucleoside analogs	Lamividine	Antivirals	(Jung *et al.*, 2008, Takeda *et al.*, 2002)
Opioids	O-Desmethyltramadol	Analgesic	(Tzvetkov *et al.*, 2011)
Psychostimulant	D-Amphetamine		(Amphoux *et al.*, 2006)
Tyrosine kinase inhibitor	Imatinib	Antineoplastic	(Herraez *et al.*, 2013, Schmidt-Lauber *et al.*, 2012, Thomas *et al.*, 2004)

Table 1.2 - Endogenous compounds transported by hOCTs and hMATEs.

Category	Compound	References
Biogenic monoamines	Histamine Serotonin	(Amphoux *et al.*, 2006, Busch *et al.*, 1998, Koepsell *et al.*, 2007)
Biogenic polyamines	Agmatine Putrescine Spermidine	(Gründemann *et al.*, 2003, Sala-Rabanal *et al.*, 2013)
Cathecolamines	Dopamine Epinephrine Norepinephrine	(Amphoux *et al.*, 2006)
Ethanolamines	Choline	(Koepsell *et al.*, 2007)
Prostaglandins	Prostaglandin E2 Prostaglandin F2α	(Kimura *et al.*, 2002)
Vitamins	N-methylnicotinamide Thiamine	(Chen *et al.*, 2014, Gorboulev *et al.*, 1997, Lemos *et al.*, 2012)
Other metabolites	Creatinine L-carnitine	(Masuda *et al.*, 2006)

Table 1.3 - Xenobiotics transported by OCTs and MATEs *in vitro*.

Category	Xenobiotic	Reference
Quaternary ammonium compounds	Tetraethylammonium	(Iwai *et al.*, 2009, Zhang *et al.*, 1998)
Herbicides	Paraquat	(Chen *et al.*, 2007)
Neurotoxins	1-Methyl-4-phenylpyridinium (MPP^+) 1-Methyl-4-phenyl-tetrahydropyridine (MPTP)	(Gorboulev *et al.*, 1997, Yang *et al.*, 2001)
Alkaloids	APD-ajmalinium Berberine Nicotine	(Kim and Shim, 2006, Nies *et al.*, 2008, Van Montfoort *et al.*, 2001)
Fluorescent Dyes	4′,6-diamidino-2-phenylindole (DAPI) 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP) [2-(4-nitro-2,1,3-benzooxadiazole-7-yl)aminoethyl]trimethylammonium Ethidium	(Ahlin *et al.*, 2008, Bednarczyk *et al.*, 2000, Kim and Shim, 2006, Lee *et al.*, 2009, Mehrens *et al.*, 2000, Yasujima *et al.*, 2011)

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.*, 1997, 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.*, 1997, 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.*, 2007).

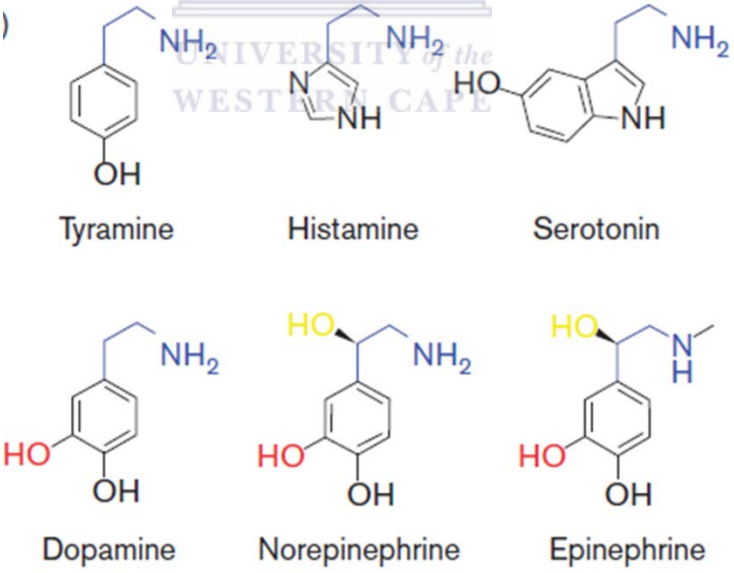

Figure 1.4 - Chemical structures of selected bio-amine substrates of hOCTs (Chen *et al.*, 2010a).

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, 2008, 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).

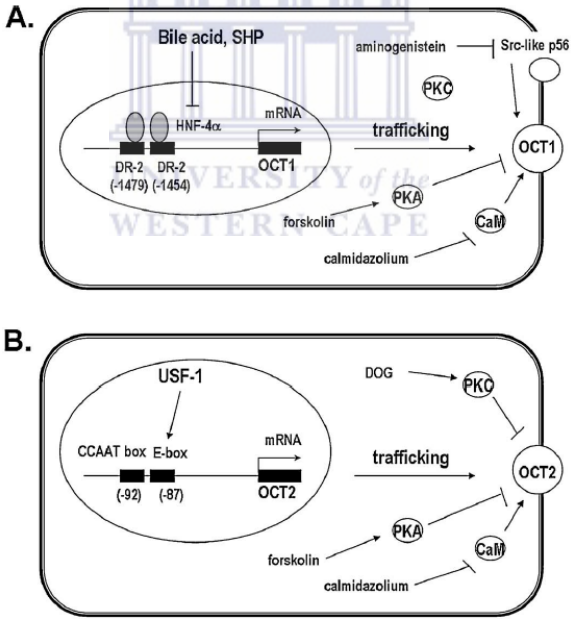

Figure 1.5 - Schematic representation of transcriptional and post-translational regulation of hOCT1 (A) and hOCT2 (B) (Choi and Song, 2008).

1.8.1.4.1. Short-Term Regulation

Mechanisms of post-translational 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 down-regulated by the PKC activator 1,2-diocanoyl-sn-glycerol (DOG).

Çetinkaya *et al.* (2003) demonstrated that hOCT2 is inhibited by PKA and phosphatidylinositol 3-kinase (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 (HNF-4α) to DNA response elements (DR-2) 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 HNF4-α 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.

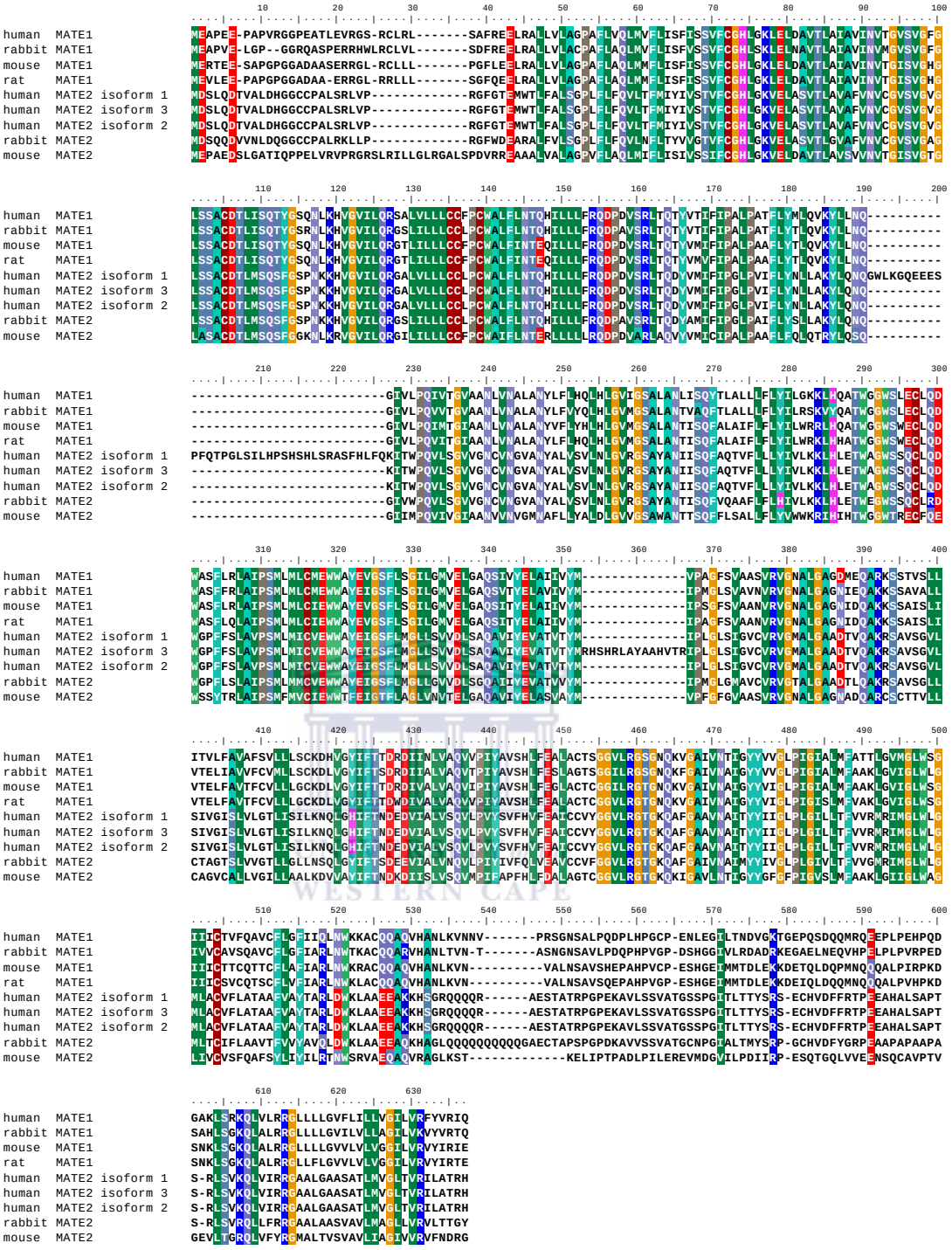

Figure 1.6 - Multiple protein sequence alignment of MATE protein sequences from four different animal species.

1.8.2. Multidrug and Toxin Extrusion 1 (MATE-1)

1.8.2.1. Structure

hMATE1 and the kidney-specific hMATE2-K 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 hMATE2-K 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 Glu-273, Glu-278, Glu-300, and Glu-389 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).

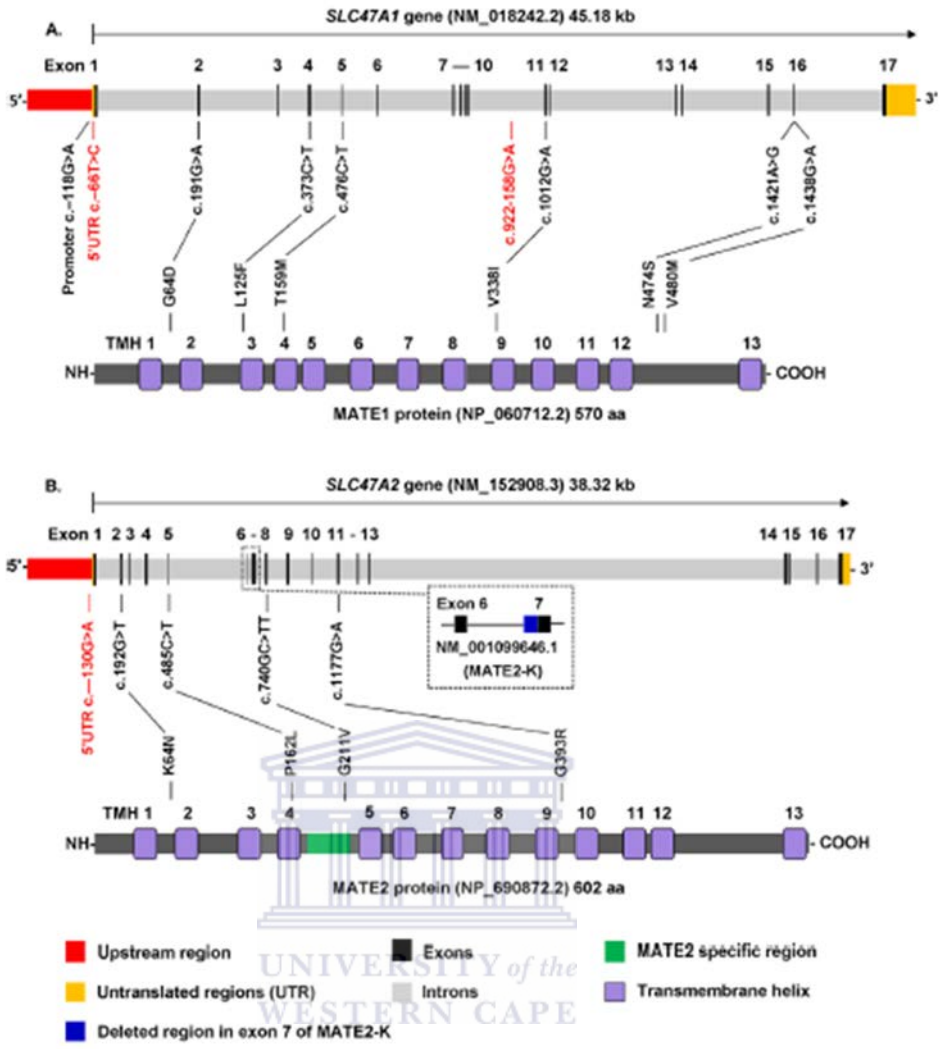

Figure 1.7 - A schematic representation of the gene organization and the primary protein structure of human (A) *SLC47A1* (hMATE1) and (B) *SLC47A2* (hMATE2-K). The diagram depicts exons (numbered 1-17) and introns of both genes together with the arrangement of transmembrane helices (TMHs) (numbered 1-13) of encoded proteins (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.*, 2009b). On the hand,

hMATE2-K is primarily expressed in the brush-border membrane of renal proximal tubules (Masuda *et al.*, 2006).

hMATE1 and hMATE2-K mediates the H^+-coupled electroneutral exchange of tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium (MMP^+), two prototypical organic cation substrates of renal and hepatic H^+-coupled organic cation antiporters (Koepsell *et al.*, 2007, 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 hMATE2-K 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 Sp1-binding 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 NF-E2-related factor 2 (NrF2) were able to alter hMATE1 or hMATE2 function.

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

1.9.1. Metformin and type-2 diabetes

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 favorable effect on hyperglycemia, metformin also has other beneficial effects. Firstly, metformin is able to exert its glucose-lowering actions with a low risk of hypoglyceamia. Secondly, it reduces the likelihood of developing macrovascular (hypertension and atheroscelorosis) 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 anti-diabetic therapeutic experience adverse events such as diarrhea 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 glycemic response (Reitman and Schadt, 2007). This variation in glycemic 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 (Matthijs L Becker *et al.*, 2009).

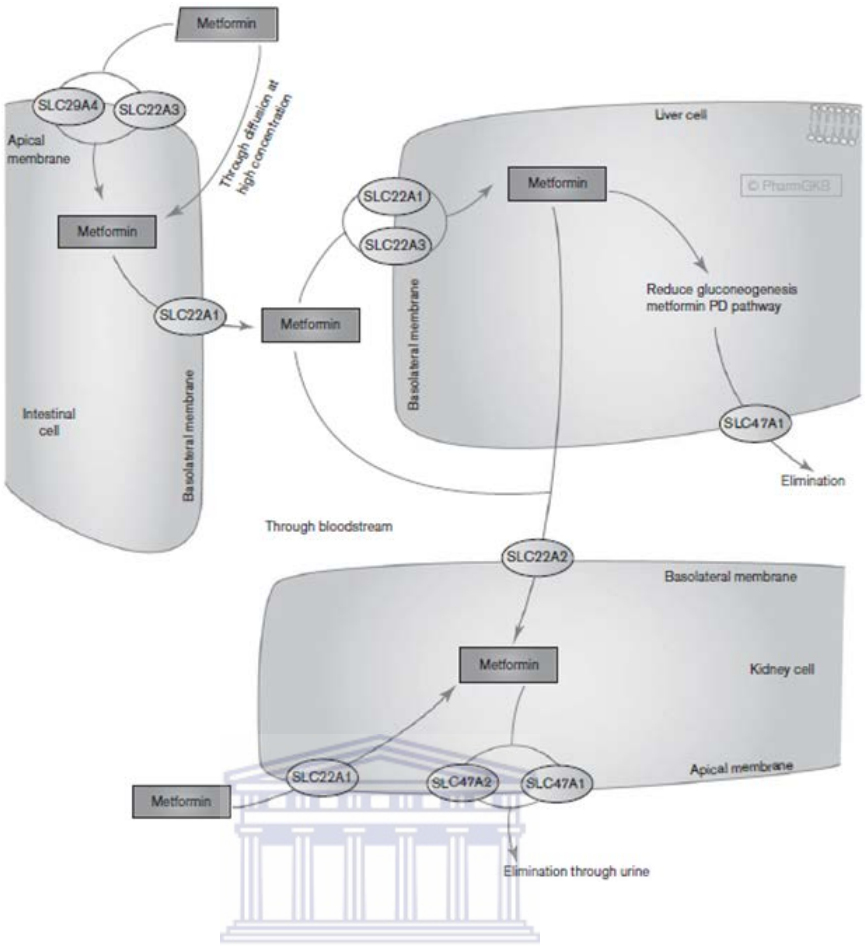

Figure 1.8 - Pharmacokinetics pathway of metformin (Gong *et al.*, 2012).

1.9.2. Implication of the Genetic Polymorphisms in OCTs on the Pharmacokinetic and/or Pharmacodynamic Profiles of Metformin in Humans

Recent studies suggest that inter-patient variability in response to metformin therapy could be related to polymorphisms in the organic cation transporter (OCT) genes and/or the multidrug and toxin extrusion (MATE) genes.

Shikata *et al*. (2007) and Shu *et al*. (2007) showed in their respective studies that hOCT1 is an important determinant of the therapeutic action of metformin and that genetic variation in the *SLC22A1* gene may contribute to variation in therapeutic response to the drug, presumably by decreasing the hepatic uptake of the drug

(Shikata *et al.*, 2007, Y Shu *et al.*, 2007). Furthermore, Shu *et al* showed that deletion of *Slc22a1* in mouse liver reduced metformin effects on 5' adenosine monophosphate-activated protein kinase (AMPK) phosphorylation and gluconeogenesis; as a consequence, the glucose-lowering effect of metformin was abolished. In addition, they demonstrated that four loss-of-function polymorphisms in *SLC22A1* in a study of 20 normal glucose-tolerant individuals resulted in a reduction of the effect of metformin on response to oral glucose (Y Shu *et al.*, 2007). Subsequently, it was shown that individuals carrying the loss-of-function hOCT1 polymorphisms had higher serum metformin concentrations, suggesting that this may be due to reduced hepatic uptake of the drug (Shu et al, 2008).

Non-synonymous genetic variants in hOCT1, implicated in reduced activity in assays done *in vitro*, have been identified largely in populations of European ancestry. However, hOCT1 is highly polymorphic in ethnically diverse populations. Tzvetkov *et al*. (2009) found in their study an increase in renal metformin excretion in individuals with amino acid variants of hOCT1 with low or missing transport which is in apparent contradiction to the findings of Shu *et al* (Shu *et al.*, 2008).

Genetic variations in *SLC22A2* are associated with decreased renal excretion and increased plasma concentrations of metformin (Shikata *et al.*, 2007, Song *et al.*, 2008a, Song *et al.*, 2008b, Wang *et al.*, 2008). Furthermore, it was shown that coadministration of cimetidine resulted in the inhibition of renal tubular secretion of metformin and a concomitant increase in plasma levels of metformin. In addition, it was also demonstrated that drug-drug interactions between metformin and cimetidine depend on genetic polymorphisms in the hOCT2 gene. However, in contrast Tzvetkov *et al*. (2009) concluded in their study that there was not a

statistically significant association of genetic variants in hOCT2 with the renal elimination of metformin (Tzvetkov *et al.*, 2009). This was consistent with the findings of previous studies which did not find any relationship between non-synonymous variations of hOCT2 and metformin uptake *in vitro* or the glucose-lowering effect of metformin (Leabman *et al.*, 2002, Shikata *et al.*, 2007). Moreover, Leabman *et al* (2002) concluded that selection has acted against amino acid changes in hOCT2, suggesting that hOCT2 is relatively intolerant of non-synonymous changes (Leabman *et al.*, 2002).

Metformin has also been identified as a good substrate of hMATE1 and hMATE2-K proteins. The hMATE1 protein is expressed in the bile canalicular membrane of hepatocytes and in the renal epithelium, whereas hMATE2-K is expressed primarily in the renal epithelium. Based on the tissue distributions of these proteins it is believed that they play an important role in the elimination of metformin into the bile (hMATE1) and urine (hMATE1 and hMATE2-K). Moreover, hMATE1 is considered to play an important role in the pharmacokinetics and pharmacodynamics of metformin (ML Becker *et al.*, 2009). Furthermore, polymorphisms in the *SLC47A1* gene were associated with a reduction in A1C level, consistent with a reduction in hMATE1 transporter activity (Matthijs L Becker *et al.*, 2009). In addition, zu Schwabedissen *et al* showed that coordinate function of hMATE1 with hOCT2 likely contributes to the vectorial renal elimination of organic cationic drugs and that altered activity of hMATE1, whether by drugs or polymorphisms, should be considered as an important determinant of renal cationic drug elimination (zu Schwabedissen *et al.*, 2010).

1.9.3. hOCT1 transcript levels and SNPs as predictors of response to imatinib in CML

Chronic myeloid leukemia (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 *BCR-ABL* gene which is then translated into the BCR-ABL 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.

1.10. 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.*, 2009b). The anti-histamine cimetidine is known to cause DDIs with OCs in the kidney, and a previous study showed that the coadministration 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 hMATE1-mediated 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 hMATE2-K (Minematsu *et al.*, 2010). They found that imatinib, nilotinib, gefitinib, and erlotinib exerted selectively potent inhibitory effects on hMATE1, hOCT3, hMATE2-K, and hOCT1, respectively. Furthermore, they found that compared to the reference hOCT1, the M420del variant was more sensitive to drug inhibition erlotinib.

1.11. OCTs and Cancer

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 anti-cancer 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 leukemia (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 down-regulation of *SLC22A3* as a mechanism responsible for cisplatin accumulation in cervical adenocarcinoma cells. Expression of hOCT3 in kidney carcinoma cell lines increases chemosensitivity to the antineoplastics 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 down-regulation of *SLC22A1* expression in HCC is associated with advance tumour stages and a worse patient survival rate. Down-regulation of *SLC22A1* expression was also associated with tumor progression and reduced patient survival in human cholangiocellular carcinoma (CCA) (Lautem *et al.*, 2013). Moreover, the down-regulation 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 tumor and normal human pancreas tissues which may modify the outcomes of patients treated with nucleoside analogs- and platinum containing regimens.

A common variant in 6q26-q27 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 6q26-27 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).

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 pharmacogenetic 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/pharmacogenetic 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, 2008, Daar and Singer, 2005).

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/pharmacogenetics in sub-Saharan Africa, and for that matter South Africa, is to ascertain the extent of the genomic diversity in our under-studied 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. 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 oligospecific (specialized for the translocation of specific metabolic or nutritional compounds), polyspecific transporters accept compounds with different sizes and molecular structures. These polyspecific 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 polyspecific 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 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 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 under-studied. Thus, little or no information is available on the extent of genetic variation in *OCT* and *MATE* genes within African populations. Although several populations from West, East, and Central Africa are included in the 1000 Genomes and HapMap 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 pharmacogenetic 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 Xhosa population of South Africa.

We have developed and optimized eight SNaPshot™ multiplex genotyping systems covering the 78 previously identified SNPs in *SLC22A1-3*, and *SLC47A1*. The *SLC22A1* systems were subsequently used to genotype 148 Xhosa individuals, which is described in **Chapter 2**. The allele and genotype frequencies obtained were then compared to other populations to assess the amount of variation between the Xhosa population and two other African populations, the Yoruba of Nigeria and Luhya of Kenya, African Americans, Caucasians, and Asians. In addition, the genotypic

information was used to establish haplotype structures for the Xhosa population in the *SLC22A1* gene which can potentially be used in future pharmacogenetic studies. In **Chapter 3** we expanded the study to investigate genotype and allele frequency distributions of 20 nonsynonymous SNPs of *SLC47A1*, the gene that codes for the H^+/organic cation efflux transporter hMATE1, which is co-localized with the uptake OCTs hOCT1 (liver) and hOCT2 (kidney) and facilitates the elimination of cationic drugs and other xenobiotics from the kidney into the urine. **Chapter 4** describes the genotyping of 20 nonsynonymous SNPs of *SLC22A2*, the gene coding for the kidney-specific hOCT2 which is a hOCT1 paralogue and a known site of DDIs. The hOCT1 and hOCT2 paralogue hOCT3 has been identified as a biomarker in several cancers and is also involved in the transport of various antineoplastics. The extent of genetic variation within the *SLC22A3* gene, encoding hOCT3, was assessed by genotyping previously identified 18 nonsynonomous as described in **Chapter 5.** In order to determine whether the Xhosa participants harboured any novel genetic polymorphisms, we embarked on sequencing approximately 500bp of the proximal promoter region and all 11 exons plus flanking regions of *SLC22A2* in 96 individuals as described in **Chapter 6.** The main conclusions of the project were summarized in **Chapter 7,** and few prospects for the pharmacogenetics of OCTs and MATEs in the indigenous African populations residing in South Africa were also presented.

CHAPTER 2

Genetic Polymorphisms and Haplotype Structure of *SLC22A1* in the Xhosa Population

2.1. Abstract

Human organic cation transporter 1 is primarily expressed in hepatocytes and mediates the electrogenic transport of various endogenous and exogenous compounds, including clinically important drugs. Genetic polymorphisms in the gene coding for human organic cation transporter 1, *SLC22A1*, 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 nineteen nonsynonymous and one intronic *SLC22A1* single nucleotide polymorphisms were determined in 148 healthy Xhosa participants from South Africa, using a SNAPshot™ multiplex assay. In addition, haplotype structure for *SLC22A1* was inferred from the genotypic data. The minor allele frequencies for S14F (rs34447885), P341L (rs2282143), V519F (rs78899680), and the intronic variant rs622342 were 1.7%, 8.4%, 3.0%, and 21.6%, respectively. None of the participants carried the variant allele for R61C (rs12208357), C88R (rs55918055), S189L (rs34104736), G220V (rs36103319), P283L (rs4646277), R287G (rs4646278), G401S (rs34130495), M440I (rs35956182), or G465R (rs34059508). In addition, no variant alleles were observed for A306T, A413V (rs144322387), M420V (rs142448543), I421F, C436F (rs139512541), V501E, or I542V (rs137928512) in the population. Eight haplotypes were inferred from the genotypic data. This study reports important genetic data that could be useful for future

pharmacogenetic studies of drug transporters in the indigenous Sub-Saharan African populations.

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.*, 2003). In general, genetic factors are estimated to account for 15-20% of inter-individual variations in drug disposition and responses (Choi and Song, 2008, 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).

Polyspecifc organic cation transporters (OCTs) are involved in the sodium-independent electrogenic transport of small organic cations (OCs) with different molecular structures (Koepsell *et al.*, 2007). These organic cations include clinically important drugs (metformin, cimetidine, procainamide), endogenous compounds (dopamine, norephinephrine, and toxic substrates (tetra-ethylammonium, haloperidol-derived pyridinium metabolite, 1-methyl-4-phenylpyridinium) (Gorboulev *et al.*, 1997). Based on their substrate properties and tissue distributions, human OCT1-3 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.*, 1997, Koehler *et al.*, 1997, Koepsell *et al.*, 2007). 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.*, 1997, Zhang *et al.*, 1997).

The role of hOCT1 in the clinical pharmacology of clinical therapeutics such as the anti-diabetic drug metformin, the anti-neoplastic imatinib, the anti-HIV drug lamivudine, and the serotonin receptor type antogonists tropisetron and ondansetron has been extensively researched (Yan 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.*, 2007).

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.*, 2003). 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 under-studied (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 Bantu-speaking populations such as the Xhosa, Zulu, and Sotho, which are believed to have originated approximately 3000 – 5000 years ago in West Africa between the present-day Cameroon and Nigeria (Berniell-Lee *et al.*, 2009, Lane *et al.*, 2002). The indigenous African populations potentially contain a significant amount of genomic diversity (Tishkoff *et al.*, 2009, Hardy *et al.*, 2008). These populations include the Xhosa, historically indigenous to the Eastern Cape Province of South Africa, and the second largest ethnic grouping in the country making up an estimated 8 million or 17.6% of the South African population (Drögemöller *et al.*, 2010, Warnich *et al.*, 2011).

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 pharmacogenetically 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 non-existent. Therefore, the aim of this study was to investigate the genotypic and allelic distributions of nineteen nonsynonymous and one intronic SNP(s), and to infer the haplotype structure of the *SLC22A1* gene in the Xhosa population. These SNPs include A306T, A413V, M420V, C436F, I421F, V501E, V519F, and I542V for which, to our knowledge, no population data exist in the public domain.

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 148 unrelated healthy volunteers from the Xhosa population. Ethnicity of volunteers was determined by self-report.

2.3.2. DNA extraction

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.*, 2004a).

2.3.3. SNP selection

A total of 20 OCT1 gene SNPs (19 nonsynonymous and 1 intronic) were selected for this study. SNPs were selected from the literature and the Ensembl database (http://www.ensebl.org) (Flicek *et al.*, 2012). Variants A306T, A413V, M420V, C436F, I421F, V501E, V519F, and I542V 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 **Table 2.1**, were designed to have an annealing temperature between 55°C and 60°C using Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3). To test for possible non-specific amplification, primers were aligned with the NCBI sequence databases using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast/blast-cgi). 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 2.2** and **2.3**.

Table 2.1 - 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	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

2.3.5. Multiplex PCR

All the *SLC22A2* exons and the portion of intron 9 spanning rs622342 were simultaneously amplified using the primers listed in **Table 2.1**. The PCR reactions were performed in a 20 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qaigen multiplex PCR master mix (Qaigen, 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 un-incorporated 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 NanoDrop.

Table 2.2 – *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	polydGACT tail
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

Table 2.3 – *SLC22A1* multiplex 2 single base extension primers.

NCBI (dbSNP)	Amino Acid Change	Nucleotide Change	Single Base Extension Primers (5′ to 3′)	Position Accession number (NC_000006.12)	dGACT	Size bp
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

2.3.6. Multiplex minisequencing reactions

Multiplex minisequencing was performed in a 10 μl reaction volume using 3 μl of a 1/10 dilution of purified PCR products, 0.1-0.2 μM of primers, and 5 μl of SNaPshot® 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 minisequencing products

The purified minisequencing products (1 μl) were mixed with 8.7 μl of HiDi™ formamide and 0.3 μl of GeneScan-120 Liz size standard (Applied Biosystems) 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 (Applied Biosystems). 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 148 healthy Xhosa individuals between the ages of 18 and 61 years. There were 80 (54%) female and 68 (46%) 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.

The genotype and allele frequencies of the 20 OCT1 gene SNPs investigated in the 148 Xhosa subjects are summarized in Table 4. The allelic frequency of each SNP was in HWE ($p>0.05$), except for rs622342. Sixteen out of the nineteen investigated nonsynonymous SNPs were monomorphic in the Xhosa 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 wild-type (CC), heterozygote (CT) and homozygote (TT) were 96.6%, 3.4% and 0.0%, respectively. The MAF observed for S14F was 1.7%. The P341L variant genotype frequencies, on the other hand, for homozygote wild-type (CC), heterozygote (CT) and homozygote (TT) were 83.1%, 16.9% and 0.0%, respectively. The V519F variant genotype frequencies for homozygote wild-type (CC), heterozygote (CT) and homozygote (TT) were 93.9%, 6.1% and 0.0%, respectively.

Table 2.4 - Genotype and allele frequencies of OCT1 *(SLC22A1)* gene SNPs in 148 healthy Xhosa individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			
		Genotype	%	95% CI	Allele	%	95% CI	HWE (P)
S14F	rs34447885	CC CT TT	96.6 3.4 0.0	92.0 – 98.8 1.2 – 8.0 0.0 – 3.1	C T	98.3 1.7	96.3 – 99.1 0.9 – 3.7	0.834
R61C	rs12208357	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	
C88R	rs55918055	TT TA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	T A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
S189L	rs34104736	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	
G220V	rs36103319	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	
P283L	rs4646277	CC CT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
P341L	rs2282143	CC CT TT	83.1 16.9 0.0	74.6 – 80.9 12.8 – 19.1 0.0 – 3.1	C T	91.6 8.4	87.0 – 93.7 6.3 -13.0	0.261
G401S	rs34130495	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	
M440I	rs35956182	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	
G465R	rs34059508	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	
V519F	rs78899680	GG GT TT	93.9 6.1 0.0	88.6 – 96.9 3.1 – 11.4 0.0 – 3.1	G T	97.0 3.0	94.2 – 98.5 1.5 – 3.7	0.703
Intronic SNP	rs622342	AA AC CC	64.2 28.4 7.4	54.7 – 70.3 22.3 – 36.9 6.1 – 16.2	A C	78.4 21.6	72.2 – 81.8 18.2 – 23.0	0.048
R287G	rs4646278	CC CG GG	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C G	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
I542V	rs137928512	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	
M420V	rs142448543	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	

A413V	rs144322387	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
I421F	rs151333280	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
C436F	rs139512541	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
V501E	rs143175763	TT TA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	T A	100.0 0.0	98.4 – 100.0 0.0 – 1.6
A306T	COSM164365	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

The MAF of a selected number of the investigated *SLC22A2* gene SNPs in different ethnic groups are summarized in **Table 2.5** and depicted in **Figure 2.1**. *SLC22A1* SNP variants R61C (rs12208357), C88R (rs55918055), S189L (rs34104736), G401S (rs34130495), and G465R (rs34059508) were not observed in the Xhosa, Sub-Saharan or Asian populations. However, it was observed in Caucasian populations. Two *SLC22A1* SNP variants, S14F and V519F, were only observed in the Xhosa and the other Sub-Saharan populations, but not in the Asian or Caucasian populations. Inferred haplotypes are listed in **Table 2.6**.

Table 2.5 - Comparison of MAF of *SLC22A1* gene SNPs of the Xhosa population to other ethnic groups.

dbSNP ID	Amino acid change	Minor Allele	Minor Allele Frequency (%)							
			Xhosa[a]	Luhya[b]	Yoruba[b]	African-American[c]	Japanese[d]	Chinese-Han[b]	Caucasian-Finish[b]	Caucasian-American[e]
rs34447885	S14F	T	1.7	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	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
rs36103319	G220V	T	0.0	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	8.4	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	0.0	0.0	0.0	0.5	0.0	0.0	2.7	0.0
rs34059508	G465R	A	0.0	0.0	0.0	0.0	0.0	0.0	1.1	4.0
rs78899680	V519F	T	3.0	2.0	6.0	ND	ND	0.0	0.0	ND
rs622342	Intronic	C	23.0	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)

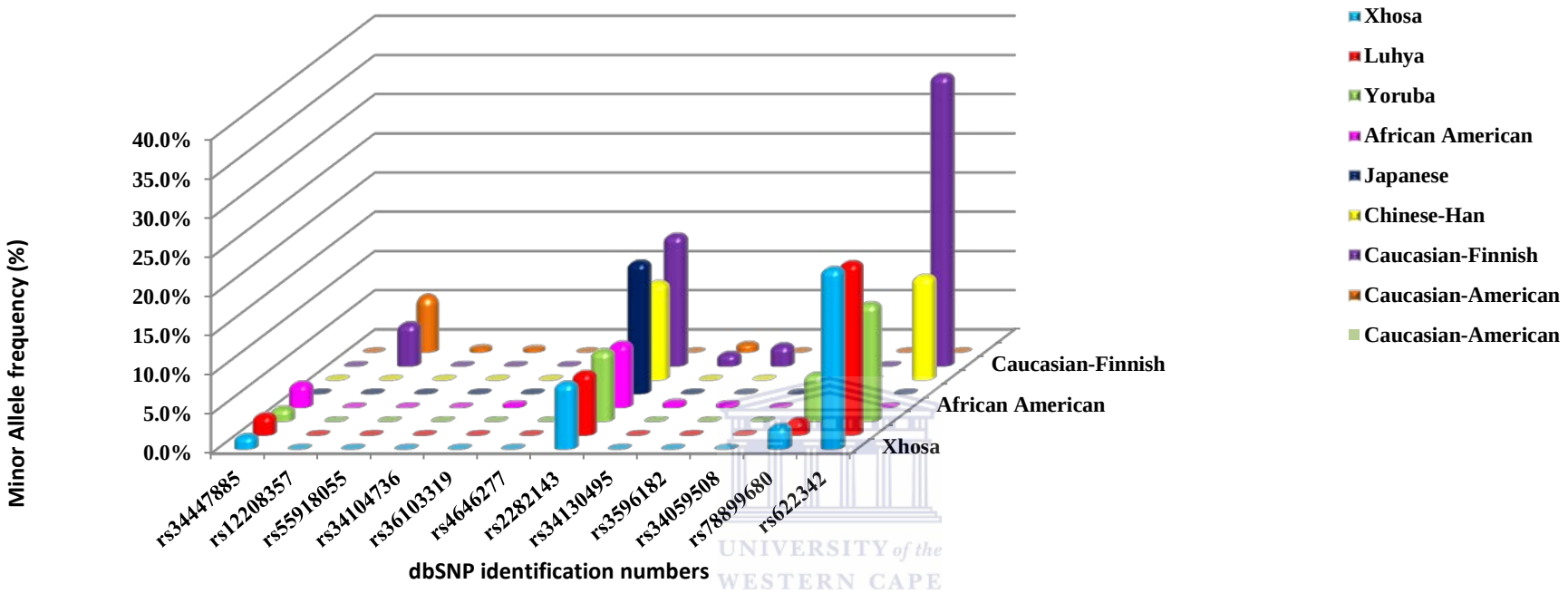

Figure 2.1 - Allele frequencies of selected *SLC22A1* SNPs in the Xhosa population compared to other African and world populations.

Table 2.6 - Haplotype structure defined by 20 SNPs in the *SLC22A1* gene in the Xhosa population.

Haplotype No.	Haplotypes [a]	Frequency %
Haplotype *1	CCTCGCCGCGCAAGGAGTGA	69.4
Haplotype *2	CCTCGCCGCGCAAGGAGTTA	2.8
Haplotype *3	CCTCGCCGCGCAAGGCGTGA	17.6
Haplotype *4	CCTCGCCGTGCAAGGAGTGA	5.0
Haplotype *5	CCTCGCCGTGCAAGGCGTGA	3.2
Haplotype *6	CCTCGCCGTGCAAGGCGTTA	0.2
Haplotype *7	TCTCGCCGCGCAAGGAGTGA	1.1
Haplotype *8	TCTCGCCGCGCAAGGCGTGA	0.6
	Total	**99.9**

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

2.5. Discussion

Single nucleotide polymorphisms in hOCT1 have been increasingly recognized as a possible mechanism explaining inter-individual variation in drug response (Leabman *et al.*, 2003). In this study we determined the allelic frequency of 20 SNPs in the hOCT1 gene of 148 healthy individuals of the Xhosa population of South Africa, and the data was compared with other published studies. No polymorphisms were observed for the Xhosa population for sixteen out of the twenty SNPs investigated in this study. In a previous study aimed at the development of male specific genotyping systems for use in sexual assault cases in South Africa, low levels of polymorphism were also observed for the Xhosa population (Leat *et al.*, 2004a, Leat *et al.*, 2007, Leat *et al.*, 2004b).

hOCT1 carrying the S14F (rs34447885) substitution was previously shown to exhibit an increased uptake of the prototypical organic cation MPP^+ (Shu *et al.*, 2003). However, in a subsequent study by Shu *et al.* (2007) it was shown that the S14F variant displayed a reduced uptake of the anti-diabetic drug metformin which, was attributed to a reduction in the transporter's V_{max} for metformin (Y Shu *et al.*, 2007). The MAF of S14F (rs34447885) for the Xhosa population (2.0%) was similar to that of African-Americans (3.0%) (Shu *et al.,* 2003) and of two other sub-Saharan African populations, the Luhya in Webuye, Kenya (3.0%) and the Yoruba in Ibadan. Nigeria (2.0%). However, the MAF was significantly higher than that observed in Caucasians (0.0%) and Asians (0.0%). Therefore, it is possible to expect that drugs which are substrates of hOCT1 could have different response profiles in the Xhosa population compared to Caucasian and Asian populations.

Previous studies have found that hOCT1 R61C (rs12208357) and G401S (rs34130495) variants showed reduced transport of the prototypical organic cation MPP^+ (Shu *et al.*, 2003). In addition, it was shown that these variants exhibited reduced transport of metformin (Y Shu *et al.,* 2007). Furthermore, the R61C variant has been reported to be strongly correlated with low hOCT1 protein expression in liver tissues of a 150 Caucasian subjects (Nies *et al.*, 2009). Moreover, these reduced-function variants were associated with an increase in the renal clearance of metformin (Tzvetkov *et al.,* 2009). Both these variants are frequently observed in Caucasian populations with MAF of 7.2% and 4%, respectively (Shu *et al.*, 2003). In contrast, none of these variants were observed for the Xhosa, Luhya, Yoruba, African-Americans or any of the Asian populations.

In *Xenopus laevis* oocytes expression systems, the uptake of the prototypical organic cation MPP^+ by the C88R (rs55918055) variant transporter was reduced to 1.4% compared with the reference, whereas serotonin uptake was reduced to only 13% of the wild-type (Kerb *et al.*, 2002). The MAF for C88R in a Caucasian population was observed at 6.2% by Kerb *et al* (Kerb *et al.*, 2002), compared to 0.0% for the Xhosa population in this study.

The hOCT1 variants G220V (rs36103319) and G465R (rs34059508) were first identified as non-functional variants. The G220V variant has thus far only been observed in the African American population with MAF of 0.5%, whereas, the G465R was only observed in the Caucasian population at a MAF of 4.0%. Moreover, G465R was associated with reduced localization at the basolateral membrane (Y Shu *et al.*, 2007). However, none of these non-functional variants were observed in this study for the Xhosa population. These variants were also not observed in other African populations or in any of the Asian populations.

The allele frequency of P341L (rs2282143) in the Xhosa population (8.4%) was similar to those of other Sub-Saharan African populations, lower than the Asian populations, and significantly higher than that of the Caucasian populations (Table 5). Functional transport assays conducted *in vitro* have shown that the P341L variant results in a decrease rate of MPP^+ transport, and has no effect on the transport of the anti-diabetic drug metformin (Sakata *et al.*, 2004, Y Shu *et al.*, 2007). Thus, impaired transport activities related to the P341L SNP may differ between Africans, Asians, and Caucasians, with consequent effects on the pharmacokinetics/pharmacodynamics of certain substrates (Kang *et al.*, 2007).

The P283L (rs4646277) variant was first described in a Japanese population and was shown to have reduced transport activity despite similar protein expression levels of the plasma membrane (Takeuchi *et al.*, 2003, Sakata *et al.*, 2004). The P283L variant was subsequently also found in other Asian populations with an allele frequency of 1.3% in a Korean population and did not differ significantly from those of Chinese and Vietnamese populations (Kang *et al.*, 2007).This variant was however not observed in the Xhosa population or any of the other African populations nor in any of the Caucasian populations **(Figure 2.1)**.

The hOCT1 Met420 deletion (rs72552763) that occurs in exon 7 is a common variant with a MAF of 18.5% in Caucasians and 5% in African Americans, respectively (Goswami *et al.*, 2014, Shu *et al.*, 2003). The expression of this variant in HEK293 cells resulted in reduced hOCT1 function with a concomitant reduction in metformin uptake by altering the kinetics (decreasing V_{max}) of metformin (Shu *et al.*, 2008). In addition, the Met420del variant is also associated with an increased probability of imatinib failure in patients with CML (Giannoudis *et al.*, 2013). However, this variant was not observed in the current study population.

The intronic SNP rs622342 was first reported by Becker *et al.* (2009) in a group of Dutch incident metformin users with a MAF of 37.0% (ML Becker *et al.*, 2009). In contrast, the MAF for this variant in the Xhosa population was lower (21.6%). Moreover, Becker *et al*. (2009) concluded that an association existed between genetic variation in the gene encoding for the hOCT1 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 (ML Becker *et al.*, 2009). In a subsequent study, by the same group, it was shown that the

effect of the hMATE1 rs2289669 polymorphism on the glucose lowering effect of metformin was larger in patients with the hOCT1 rs622342 CC genotype than those with the AA genotype (Becker *et al.*, 2010).

In the present study, the nonsynonymous SNP V519F (rs78899680) was also genotyped and the observed MAF was 3.0%. This value was higher than that of the Luhya (2.0%), a population from Eastern Africa, and lower than that of the Yoruba (6.0%) of Western Africa (Table 5). However, this variant was not observed in any of the Caucasian or Asian populations, indicating that it may 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.

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 pharmacogenetic 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). In this study, the haplotype structure defined by 20 SNPs in the *SLC22A1* gene was inferred for the investigated population. The most frequently observed haplotypes were CCTCGCCGCGCAAGGAGTGA (69.4%), CCTCGCCGCGCAAGGCGTGA (17.6%), and CCTCGCCGTGCAAGGAGTGA (7.0%).

Although Africa is the continent where the burden of disease is the heaviest, research and clinical trials are predominantly performed on Caucasian and Asian populations. This contributes to poor treatment response and occurrence of adverse drug reactions in the genetically diverse African populations. Thus, hOCT1variant alleles which are commonly/only found in African populations will/may have a profound impact on

organic cationic drug transport efficacy and toxicity. 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 pharmacogenetic information specific for African populations.

2.6. Conclusions

To our knowledge, this is the first study that investigated the allele and genotype frequency distributions of SNPs in the *SLC22A1* gene of the Xhosa 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* gene found in Caucasian and Asian populations are absent from the Xhosa population. We have shown that, although MAF observed for the Xhosa 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. 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*, for the indigenous populations of South Africa. The uptake transporters hOCT1 and hMATE1 are co-localized in the liver and function cooperatively in the elimination of organic cation substrates such as metformin from hepatocytes. Thus, **Chapter 3** will focus on the investigation of

allele frequency distribution of potential deleterious *SLC47A1* genetic variants in the Xhosa population.

CHAPTER 3

Assessment of Genetic Variations within the *SLC47A1* Gene of the Xhosa Population

3.1. Abstract

Multidrug and toxin extrusion 1 (MATE1) is a recently identified organic cation/H^+ exchanger, localized in the apical membrane of proximal renal tubules, which mediates the cellular elimination of organic cations into the renal lumen. These organic cations include clinically important drugs such as metformin, oxaliplatin, and cimetidine. Moreover, genetic polymorphisms of *SLC47A1*, the pharmacogenetically relevant gene encoding hMATE1, have been implicated in reduced transport or accumulation to cytotoxic levels of these drugs *in vitro.* However, little or no information is available on the minor allele frequency distribution of known *SLC47A1* coding SNPs in the sub-Saharan African populations. Thus, the aim of this study was to determine the baseline minor allele frequency distribution of 20 known coding SNPs in the *SLC47A1* gene of 148 Xhosa individuals residing in Cape Town, South Africa. This study did not identify any of these known *SLC47A1* coding SNPs in the Xhosa individuals that participated in this study. This study lays the foundation for future association studies between *SLC47A1* variations and treatment outcomes in the Xhosa population. However, this study has inherent limitations and was not exhaustive with regards to known *SLC47A1* polymorphisms. Furthermore, whole genome or exome sequencing may reveal novel SNPs in the Xhosa and other sub-Saharan African populations, which may have been missed with the current genotyping strategy.

3.2. Introduction

Multidrug and toxin extrusion (MATE) proteins are recently discovered secondary active transporters responsible for the efflux of organic cations (OCTs) from cells. MATEs are widely distributed and have been cloned from various living organisms, including prokaryotes, plants, and mammals (Otsuka *et al.*, 2005). This widespread distribution of MATEs coupled with their capacity to transport a wide variety of endo-/exogenous substrates underlines the importance of these transporters in physiological and/or pharmacological processes such as pharmacokinetics, resistance to antimicrobials in bacteria, resistance to chemotherapeutic agents in tumour tissues, and hormone secretion (Staud *et al.*, 2013).

hMATE1, consisting of 570 amino acid residues, is encoded by the *SLC47A1* gene which is located on chromosome 17p11.2 (Otsuka *et al.*, 2005). MATE transporters are primarily expressed in the kidney and liver, and they are localized at the apical membrane of the renal tubules and bile canaliculi, and transport organic cations (OCs) with an oppositely directed H^+ gradient as a driving force (Masuda *et al.*, 2006, Tsuda *et al.*, 2007). However, a recent study found that hMATE1/hMATE isoforms are also expressed in the human placenta, albeit with considerable inter- and intra-individual variability (Ahmadimoghaddam *et al.*, 2013) Substantial evidence has accumulated that implicates hMATE1, hMATE2, and its kidney-specific homolog hMATE2-K as critical components in the luminal efflux of OCs from renal proximal tubules into urine (Komatsu *et al.*, 2011, Masuda *et al.*, 2006, Tsuda *et al.*, 2009a, Watanabe *et al.*, 2010). *In vitro* studies have shown that MATE1 and MATE2-K are involved in the transport of prototypical OCs such as tetraethylammonium bromide (TEA), the neurotoxin 1-methyl-4-pyridinium (MPP^+),

the histamine antagonist-receptor inhibitor cimetidine, the antidiabetic drug metformin, the antiarrhythmic drug procainamide. In addition, MATE1 can also effectively transport the zwitterionic β-lactam antibiotics cephalexin and cephradine and the anionic compounds estrone sulphate, acyclovir, and ganciclovir (Tanihara *et al.*, 2007, Terada *et al.*, 2006). Because MATEs and OCTs cooperate in transcellular passage, considerable overlap in substrate specificity must exist between these transporters (Iwata *et al.*, 2012).

Mammalian MATE1, similar to other MATE family members have a predicted protein fold composed of 12 transmembrane helices (TMHs) (He *et al.*, 2010, Zhang *et al.*, 2012). Zhang *et al* in their study showed that the human orthologue of MATE, similar to its rabbit and mouse paralogues, has an extacelluar C-terminus, consistent with the presence of 13 TMHs (Zhang and Wright, 2009, Zhang *et al.*, 2012). Moreover, Zhang *et al.* (2012) concluded that the terminal 13th TMH is not required for the functional activities of human, rabbit, and mouse MATE1, and that ligand binding of truncated proteins consisting of 12 TMHs is essentially normal. This supports the general view that the functional core structure of MATE proteins, including mammalian MATEs, is comprised of 12 TMHs and that the 13th TMH is merely influences transporter turnover activity (Zhang *et al.*, 2012). Combined *in vitro/in silico* and quantitative structure-activity relationship (QASAR) models suggest that MATE preferentially bind large, lipophilic, and positively charged molecules (Astorga *et al.*, 2012, Wittwer *et al.*, 2013).

In animal models of human diseases, Mate1 expression was decreased in renal failure and increased in metabolic acidosis, suggesting pathological conditions may affect MATE1 expression and resultantly the pharmacokinetics and

pharmcodynamics of its substrates (Gaowa *et al.*, 2011, Nishihara *et al.*, 2007). Becker *et al.* (2009) demonstrated that genetic variation in the *SLC47A1* gene was associated with the glucose-lowering effect of metformin (Matthijs L Becker *et al.*, 2009). Ha Choi *et al.* (2009) found that a common promoter variant may affect the expression of MATE1 in the kidney, and may ultimately result in variation of drug disposition and response (Choi *et al.*, 2009).

In addition to genetic polymorphisms, adverse drug-drug interactions (DDIs) can also affect the pharmacokinetics and/or pharmacodynamics of hMATE substrate drugs. In a study by Tsuda *et al.* (2009) double-transfected Madin-Darby canine kidney (MDCK) cells stably expressing both hOCT2 and hMATE transporters were used as an *in vitro* model of proximal tubular epithelial cells to assess the interaction of the histamine-receptor antagonist, cimetidine with the biguanide antidiabetic, metformin (Tsuda *et al.*, 2009b). Contrary to the findings of previous studies, they found that cimetidine showed a higher affinity for hMATEs than hOCT2. Subsequently, Ito *et al.* (2012) showed that competitive inhibition of the luminal efflux by hMATE1, and not the basolateral uptake by hOCT2, is the likely mechanism underlying the pharmacokinetic DDIs caused by cimetidine in the kidney (Ito *et al.*, 2012). Furthermore, in a study by Grün *et al.* (2013) the antibiotic trimethoprim significantly reduced metformin elimination resulting in increased exposure to the antidiabetic (Grun *et al.*, 2013). The anti-malarial drug pyrimethamine is a potent inhibitor of both hMATE1 and hMATE2-K activity (Ito *et al.*, 2010).

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). 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 Xhosa population, historically indigenous to the Eastern Cape Province, is the second largest ethnic group in South Africa, and comprises approximately 17.6% (~8 million) of the total population (Drögemöller *et al.*, 2010). Previous studies have shown that these populations harbour unique genotype and allele frequencies for pharmacogenetically 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 the indigenous South African populations. Therefore, the aim of this study was to determine the baseline genotype and allele frequency distributions of 20 known *SLC47A1* coding SNPs in 148 Xhosa 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.*, 2004a). A total of 20 *SLC47A1* 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 *SLC47A1* exons and flanking regions were designed using Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3) and are listed in **Table 3.1**. To test for possible non-specific amplification, primers were aligned with the NCBI sequence databases using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast/blast-cgi). 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 3.2** and **3.3**.

Table 3.1 - MATE-1 multiplex PCR primers for the amplification of *SLC47A1* exons and flanking regions.

Location	Forward primer 5′ to 3′	Reverse primer 5′ to 3′	Nucleotide position	Amplicon size (bp)
Exon 2	CCCAGGTGACAGTGTGAGAC	CAGCAGAAGGCAAACACAGA	19542097 - 19542550	454
Exon 3	GGTCCAGGCAGCTAACAAAG	CTGGCTGCTGAACTCTTCCT	19546291 - 19546599	309
Exon 4	TATCCAGCCAACCTGCTTCT	GGAGGGTGCTTGCAAATCTA	19547879 - 19548374	496
Exon 5	GGAAACAGCCAAGAATGGAA	GACCCCAAGAAGGGAATCTC	19549522 - 19549716	195
Exon 6	CTGAGACGACAGCCTCTGTG	CCCATTCCCAGAAAGGTACA	19551282 - 19551531	250
Exon 7	CAGTCCTTGCACTGTTGGAA	TCCATCCCTGACAGTGCTTT	19554910 - 19555456	547
Exon 8 - 10	CACGGGAAGGGATGAGTCT	GGAGATGGAGAACCAGCAGA	19555502 - 19556294	793
Exon 11 - 12	TGCTTCTCTGCACGTGTTCT	CCTCCTGGGCTCAAGAGATT	19560120 - 19560809	690
Exon 13 - 14	GGCTGGTCTCAAACTCCTGA	GCCCCCTACACTCTCTGACA	19566635 - 19567377	743
Exon 15	CCTCAGCCATGAAAGCAGAT	ATCATCTGGCCCTTCACATC	19571359 -19571748	390
Exon 16	TGGGATTACAGGTGTGAGCA	CTCACTAACAGCCCCTCCAG	19572589 - 19572928	340
Exon 17	GGGCACTCTGCGATAAGATT	CAATGCAGTCAGCACATTGA	19577166 - 19577823	658

3.3.4. Multiplex PCR

All the 17 *SLC47A1* exons and their flanking regions were simultaneously amplified using the primers listed in **Table 3.1**. The PCR reactions were performed in a 20 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qaigen multiplex PCR master mix (Qaigen, 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 minisequencing was performed in a 10 μl reaction volume using 3 μl of a 1/10 dilution of purified PCR products, 0.1-0.2 μM of primers, and 5 μl of SNaPshot™ ready reaction mix (Applied Biosystems). Sequence cycling was performed according to the instructions of the manufacturer. Post-extension treatment was done as described in **Chapter 2**

Table 3.2 – Multiplex 1 of *SLC47A1* single-base extension primers for SNaPshot™ minisequencing reactions.

NCBI (dbSNP)	Amino Acid Change	Nucleotide change	Nucleotide sequence (5′ to 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	polyGACT tail
rs143217368	T75M	C>T	ACCTGGGCAAGCTGGAGCTGGATGCAGTCA	19542481	30	0
rs77474263	L125F	C>T	ATCCTGCAGCGGAGTGCGCTCGTCCTGCTC	19548051	35	5
rs143542564	S151F	C>T	TGCTGCTCTTCAGGCAGGACCCAGATGTGT	19548130	40	10
COSM218508	A193S	G>T	ATTGTACTGCCCCAGATCGTAACTGGAGTT	19555245	45	15
rs145720500	M269T	T>C	TCCTCCGCCTGGCCATCCCCAGCATGCTCA	19555862	50	20
rs149774861	D328A	A>C	TCCGGGTAGGAAACGCTCTGGGTGCTGGAG	19560249	55	25
COSM141490	A409V	C>T	TGAGGGGGAGTGGAAATCAGAAGGTTGGAG	19567145	60	30
rs147768037	G424R	G>A	CAAGTGTGGTTGCAAACATCAGCGCGATCC	19567189	65	35
rs141945405	A473T	G>A	CTGAGTTTCATTTTCCAGGCTCAGGTACAC	19572791	70	40
rs144621154	R545Q	G>A	AAGAAGACCCCCAGGAGCAGAAGCCCTCGC	19577474	75	45

Table 3.3 - Multiplex 2 of *SLC47A1* single-base extension primers for SNaPshot™ minisequencing reactions.

NCBI (dbSNP)	Amino Acid Change	Nucleotide change	Nucleotide sequence (5′ to 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	polyGACT tail
rs139122064	R482W	C>T	CACGCCAATTTGAAAGTAAACAACGTGCCT	19572819	30	0
rs145557304	Y203C	A>G	CAGCCAACCTTGTCAATGCCCTCGCCAACT	19555276	35	5
rs11551331	P148R	C>G	AGCACATCCTGCTGCTCTTCAGGCAGGACC	19548121	40	10
rs148155569	A469S	G>T	AAGCCTGATGGACTGAGTTTCATTTTCCAG	19572780	45	15
rs77138970	R118Q	G>A	ACCTGAAGCACGTGGGCGTGATCCTGCAGC	19548031	50	20
rs149729794	P186T	C>A	GCGGTGTCCTTTTTCCAGGGAATTGTACTG	19555224	55	25
rs77630697	G64D	A>G	TGATCAGCTTCATAAGCTCCGTGTTCTGTG	19542448	60	30
rs148469848	Y566C	A>G	TCTTGCTGGTGGGGATTTTAGTGAGATTCT	19577537	65	35
rs149920616	G397S	G>A	AATGCTCTCTGCCTGCAGTGCACGAGTGGT	19567108	70	40
rs141572615	V279D	T>A	TGCTGTGCATGGAGTGGTGGGCCTATGAGG	19555892	75	45

3.3.6. Electrophoresis of the minisequencing products

. The fluorescently labelled fragments were treated and separated on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems) as described in **Chapter 2**. Data analyses were performed using GeneMapper® IDX Software Version 1.2.

3.4. Results and Discussion

Our study population consisted of a 148 healthy, unrelated Xhosa individuals residing in the Cape Town Metropolitan area, South Africa. The age of the participants ranged from 18 to 61 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 *SLC47A1* gene. The results for the SNaPshot™ genotyping are summarized in **Table 3.4**. Genetic variants of the 20 SNPs assayed were absent in the Xhosa individuals analysed in this study.

Table 3.4 - Genotype and allele frequencies of MATE1 *(SLC47A1)* gene SNPs in 148 healthy Xhosa individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			HWE (P)
		Genotype	%	95% CI	Allele	%	95% CI	
G64D	rs77630697	AA AG GG	100.0 0.0 0.0	96.9.0 – 100.0 0.0– 1.3 0.0 – 1.3	A G	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
T75M	rs143217368	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	
R118Q	rs77138970	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	
L125F	rs77474263	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	
P148R	rs11551331	CC CG GG	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C G	100.0 0.0	98.4 – 100.0 0.0 -1.6	
S151F	rs143542564	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	
P186T	rs149729794	CC CA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
A193S	COSM218508	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	
Y203C	rs145557304	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	
M269T	rs145720500	TT TC CC	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	T C	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
V279D	rs141572615	TT TA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 3.1	T A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
D328A	rs149774861	AA AC CC	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	A C	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
G397S	rs149920616	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	
A409V	COSM141490	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	
G424R	rs147768037	GG GA	100.0 0.0	96.9 – 100.0 0.0 – 1.3	G A	100.0 0.0	98.4 – 100.0 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
A469S	rs148155569	GT	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
A473T	rs141945405	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
R482W	rs139122064	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
R545Q	rs144621154	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
Y566C	rs148469848	AG	0.0	0.0 – 1.3	G	0.0	0.0 – 1.6
		GG	0.0	0.0 – 1.3			

The human MATE1 is highly expressed at the apical membranes of liver hepatocytes and renal proximal tubules, and mediates the biliary and renal excretion of many cationic drugs and their metabolites (Otsuka *et al.*, 2005). Functional changes in the expression level of *SLC47A1* caused by genetic variations in the coding and noncoding regions could alter the transport activity of MATE1 and ultimately the effects of the substrates translocated by the transporter *in vivo*. The aim of this study was to determine the allelic and genotypic distribution of 20 known coding SNPs in the *SLC47A1*gene, encoding MATE1, in the Xhosa population of South Africa.

This study did not identify any of the known investigated nonsynonymous coding variations in the *SLC47A1* gene of the Xhosa individuals. However, searching the dbSNP database revealed only 60 nonsynonymous coding SNPs, with very low minor allele frequencies, in the *SLC47A1* gene. This lack of protein sequence and mutational diversity was also observed in other studies for hOCT2 and hOCT3 (Kang *et al.*, 2007, Lazar *et al.*, 2003). This absence of genetic diversity merely

demonstrates the critical role the MATE1 transporter performs in the elimination of xenobiotics and the maintenance of cellular and organismal homeostasis (Lazar *et al.*, 2003, Leabman *et al.*, 2003).

Kajiwara *et al.* (2009) identified the hMATE1 variant G64D in their study of 89 healthy Japanese individuals (Kajiwara *et al.*, 2009). This variant was also observed by Chen *et al.* (2009) in a study with 272 ethnically diverse individuals (Chen *et al.*, 2009b). Both these groups went on and demonstrated that this variant leads to a complete loss of transport activity. When Kajiwara *et al.* (2009) evaluated membrane expression levels using cell surface biotinylation, they found that the expression of the G64D variant was significantly decreased compared to that of the wild-type. This variant was included in the current study, but was not observed in the Xhosa individuals screened in this study. However, because of the limited sample size, and the low global allele frequency of this variant the presence of this variant in the Xhosa population cannot be excluded.

This study did not investigate the allele frequency distribution for known genetic variants in the proximal promoter region of the *SLC47A1* gene. However, in a study conducted by Kajiwara *et al.* (2007) the authors described a variant, g.-32G>A with a minor allele frequency of 3.7% in the *SLC47A1* proximal promoter region that showed markedly decreased promoter activity (Kajiwara *et al.*, 2007). Their study showed that the SpI transcription factor has a critical role in the basal promoter activity of the *SLC47A1* gene, and that the g.-32G>A variant reduced the binding affinity of SpI to the promoter. In a subsequent study, Ha Choi *et al.* (2009) identified novel polymorphisms in the proximal promoter of *SLC47A1*, and found that one of the polymorphisms, g.-66T>C, is associated with a significant reduction

of *SLC47A*1 promoter activity and with lower expression of MATE1 in the kidney (Choi *et al.*, 2009). They concluded that this reduced *SLC47A1* promoter activity seemed to be related to the binding of the transcription factor AP-1 which acts as an activator of transcription, and to an increased binding of the repressor AP-2_{rep} to the region containing the g.-66T>C substitution. Since these promoter variants can have a profound effect on the expression of the gene, which in turn can affect the transport activity and the pharmacokinetic and pharmacodynamic consequences of clinical drugs transported by MATE1, the determination of allele frequency distributions of known promoter SNPs and the extent of rare variants within the Xhosa population requires further investigation.

Metformin, an oral anti-diabetic drug, is one of the most frequently prescribed drugs in the treatment of type-2 diabetes. Tanihara *et al.* (2007) demonstrated that MATE1 transports metformin and could be a determinant of its efficacy (Tanihara *et al.*, 2007). The elimination of metformin from the body mainly depends on renal clearance, including excretion mediated by OCT and MATE transporters (Graham *et al.*, 2011). Genetic variations in the genes encoding the hOCT1, hOCT2, hOCT3, MATE1 and MATE2-K transporters have been associated with an altered pharmacokinetic and pharmacodynamic response to metformin (Christensen *et al.*, 2011). Becker *et al.* (2009) reported that a SNP located in the intronic region of *SLC47A1*, rs2289669G>A, was associated with the glucose-lowering effect of metformin in a group of Dutch diabetic patients (Matthijs L Becker *et al.*, 2009). However, Tzvetkov *et al.* (2009) found no association between rs2289669G>A and the renal or extrarenal clearance of orally administered metformin (Tzvetkov *et al.*, 2009). In addition, a recent study by Toyama *et al.* (2012) found that heterozygous nonsynonymous variants of both MATE1 and MATE2-K do not affect metformin

disposition in diabetic patients (Toyama *et al.*, 2012). As current knowledge in this field is still limited and contradictory, further studies are required to confirm the clinical relevance of *SLC47A1* SNPs in the pharmacokinetics and pharmacodynamics of metformin and other clinical substrates of MATE1 (Matthijs L Becker *et al.*, 2009, Tkáč *et al.*, 2013, Tzvetkov *et al.*, 2009).

3.5. Conclusions

To our knowledge this study is the first that prioritized the genotyping of known coding SNPs of *SLC47A1*, a pharmacogenetically relevant gene, in the Xhosa population. The SNPs genotyped in this study are known variants that have been observed in other populations and may not be present in the Xhosa population. Given the under-studied genomic diversity harboured within sub-Saharan indigenous African populations, the potential exist that novel and rare low frequency variations could be identified in the Xhosa population. This study also lays the foundation for future association studies between *SLC47A1* variations and treatment outcomes in the Xhosa population. The H^+/organic cation efflux transporter hMATE1 also co-operatively functions with the organic cation uptake transporter hOCT2 in the kidney where it is involved in the elimination of organic cations from the body. Therefore, **Chapter 4** will prioritize the investigation of nonsynonymous variants of the *SLC22A2* gene in the Xhosa participants.

CHAPTER 4

Genotype and Allele Frequency Distribution of 20 *SLC22A2* Single Nucleotide Polymorphisms in the Xhosa Population

4.1. Abstract

The solute carrier family of the major facilitator membrane transporters play an important role in maintaining cellular and organismal homeostasis. Organic cation transporters, which belong to the *SLC22A2* family, are polyspecific transporters that mediate the electrogenic transport of small organic cations with different molecular structures, independent of sodium gradient. The kidney-specific hOCT2, which is encoded by *SLC22A2*, is an organic cation transporter which is involved in the translocation of a diverse group of therapeutic drugs including metformin, cimetidine, procainamide, cisplatin, and lamivudine. Moreover, single nucleotide polymorphisms of *SLC22A2* are clinically significant because they can alter the transport of substrate drugs, and as such can influence the efficacy and toxicity thereof. Although it is widely accepted that African populations harbour a greater amount of genomic diversity compared to other populations, limited information is available regarding genetic polymorphisms in *SLC* genes, and for that matter the *SLC22A2* gene, of sub-Saharan African populations or on the ethnic differences between African and other populations and among African populations themselves regarding genetic polymorphisms related to impaired functional activity of hOCT2. Therefore, the initial aim of this study was to develop a multiplex SNaPshot™ genotyping assay system for 20 previously reported *SLC22A2* nonsynonymous SNPs and to assess the baseline allele frequencies of these variants in 148 Xhosa individuals residing in Cape Town, South Africa. We identified three

nonsynonymous SNPs namely, A270S, R400C, and K432Q in the population studied at minor allele frequencies of 6.1%; 3.4%; and 0.7%, respectively. The genetic variants and the allele frequencies observed in this study again highlight the fact that the distribution of these variants and their allele frequencies differ amongst African and other populations. Moreover, the distribution of genetic variants and their allele frequencies differ even among the African populations themselves. This study lays the foundation for ethnicity-specific genotype-to-phenotype correlates of treatment outcome for *SLC22A2* genetic polymorphisms and hOCT2 substrate drugs. In addition to SNPs, the haplotypes identified in this study can in future also aid in identifying associations between causative genetic variants and drug response.

4.2. Introduction

At physiological pH, approximately 40% of drugs are organic cations or weak bases which cellular uptake may be mediated by organic cation transporters (OCTs) (Neuhoff *et al.*, 2003). Polyspecific OCTs belong to the major facilitator family of solute carrier transporters, and are involved in the bidirectional transport of a variety of structurally diverse lipophilic organic cations of endogenous or xenobiotic origin across the plasma membrane (Koepsell *et al.*, 2007).

OCT-mediated transport is electrogenic and independent of sodium ion or proton gradient. Three isoforms of human OCTs exist, namely hOCT1, hOCT2, and hOCT3, which share a partially overlapping substrate spectra and have a similar membrane topology consisting of 12 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.*, 2007). However, OCTs show different

affinities to particular substrates and differ in their tissue distribution, membrane localization as well as in their regulation (Ciarimboli and Schlatter, 2005, Hayer-Zillgen *et al.*, 2002).

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.*, 1997, Koehler *et al.*, 1997, Koepsell *et al.*, 2007). 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.*, 1997, Koepsell *et al.*, 2007). The hOCT1 is predominantly localized in the liver whereas hOCT2 is mainly expressed in the basolateral membrane of kidney proximal tubules and is the major facilitator for the uptake of cationic substrates from the circulation into renal epithelial cells (Gorboulev *et al.*, 1997, Motohashi *et al.*, 2002). 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). Moreover, the 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 1-methyl-4-phenylpyridinium (MPP^+) (Burckhardt and Wolff, 2000, Gorboulev *et al.*, 1997, Okuda *et al.*, 1999,

Otsuka *et al.*, 2005, Zhang *et al.*, 1997, Zhang *et al.*, 1998). Tissue expression and membrane localization of hOCT2 are closely linked to the tissue distribution, pharmacological effects, and/or adverse effects of its substrate drugs (Aoki *et al.*, 2008).

Polymorphisms that change expression level, regulation, turnover, membrane trafficking, and/or substrate affinity of OCTs can potentially influence the therapeutic efficiency of substrate drugs and may lead to severe or even fatal adverse drug reactions (Bachtiar and Lee, 2013). Recent studies have identified several single nucleotide polymorphisms in the *SLC22A2* gene in ethnically diverse populations (Fukushima-Uesaka *et al.*, 2004, Leabman *et al.*, 2002, Kang *et al.*, 2007). However, in comparison to its liver-specific paralog, hOCT1, hOCT2 is less diverse in terms of both amino acid mutations and functional activity (Fujita *et al.*, 2006). However, single nucleotide polymorphisms identified in *SLC22A2* have been associated with changes in the pharmacokinetic/pharmacodynamics responses in substrate drugs such as metformin (Wang *et al.*, 2008).

Metformin, the biguanide anti-diabetic drug, has been identified as a superior substrate of hOCT2 (Wang *et al.*, 2008, Leabman *et al.*, 2002). Song *et al.* (2008) found that the uptake of metformin was greater in oocytes expressing the hOCT2 wildtype, than in ones expressing hOCT1 wild-type (Song *et al.*, 2008b). Moreover, transport of metformin was significantly reduced in oocytes expressing the nonsynonymous hOCT2 variants T199I (rs201919874), T201M (rs145450955), and A270S (rs316019) compared to the hOCT2 wild-type (Song *et al.*, 2008a, Song *et al.*, 2008b). In a recent study Chen *et al.* (2009) demonstrated that the A270S (rs316019) variant was associated with a greater clearance of renal metformin in

healthy volunteers of European and African ancestries (Chen *et al.*, 2009a). Moreover, Li *et al.* (2010) found that the A270S (rs316019) variant can affect the plasma lactate levels and the incidence of hyperlactacidemia (lactic acidosis), a severe and sometimes lethal side-effect of metformin treatment, in Chinese Han patients with type-2 diabetes that are on metformin therapy (Li *et al.*, 2010).

Furthermore, solute carrier transporters (SLCs), in particular OCTs, have been implicated in the cellular uptake and elimination of platinum-containing anti-cancer compounds. Burger *et al.* (2010) found that hOCT2 is a critical determinant in the uptake and toxicity of various platinum compounds, especially oxaliplatin (Burger *et al.*, 2010). This finding was supported by Sprowl *et al.* (2013) who found that hOCT2 is an important factor in oxaliplatin-induced neurotoxicity because it is responsible for the accumulation of oxaliplatin in the root ganglia cells of the nervous (Sprowl *et al.*, 2013).

In contrast to our understanding of the effects of ethnicity on drug metabolism, little information is known about ethnicity-related differences in the disposition and effects of drugs that are substrates of membrane transporters (Cropp *et al.*, 2008). Moreover, ethnic specific variations are currently not taken into account in most commercially available pharmacogenetic tests or on FDA drug labels (Ikediobi *et al.*, 2011). Although African populations harbour more genetic diversity than other populations, most pharmacogenetic studies to date have been conducted most frequently in Western European and North American Caucasians population groups only (Frazer *et al.*, 2009, Hardy *et al.*, 2008, Ikediobi *et al.*, 2011).

The *SLC22A2* gene is relevant for the pharmacokinetic disposition of a number of clinically important drugs, including those used in the treatment of type-2 diabetes,

cancer, and HIV (Burger *et al.*, 2010, Burger *et al.*, 2011, Jung *et al.*, 2008, Jung *et al.*, 2013, Kimura *et al.*, 2005a, Kimura *et al.*, 2009). Sub-Saharan Africa currently has the highest global prevalence of HIV/AIDS and rates for lifestyle diseases are rising due to rapid urbanisation (Mayosi *et al.*, 2012, Shisana *et al.*, 2009). It is estimated that approximately 60% of deaths in southern African countries are attributable to communicable diseases, whilst 30% are caused by non-communicable disorders (Coovadia *et al.*, 2009, Mayosi *et al.*, 2012). Although the role of OCTs in the pathogenesis and transport of drugs used in the treatment of these diseases are generally recognized, little or no information is available regarding genetic polymorphisms of the *SLC22A2* gene in African populations residing in southern Africa. In addition, no information is available on the inter-ethnic differences among African populations with regards to genetic polymorphisms related to impaired functional activity of hOCT2. Thus, the aim of this study was to develop a multiplex SNaPshot™ genotyping assay system for 20 previously reported *SLC22A2* nonsynonymous SNPs and to assess the baseline minor allele frequencies (MAFs) of these variants in 148 Xhosa individuals residing in Cape Town, South Africa. Secondly, to compare the MAF estimates obtained for other African, American, European, and Asian populations. Finally, to determine the haplotype structure of the *SLC22A2* gene in the Xhosa population based on the selected loci.

4.3. Materials and Methods

4.3.1. Subjects

As described in **Chapter 2**.

4.3.2. Multiplex PCR and Single-Base Extension Primer Design

Multiplex PCR and single base extension (SBE) primers, listed in **Tables 4.1 to 4.3**, were designed using Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3). To test for possible non-specific amplification, primers were aligned with the NCBI sequence databases using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast/blast-cgi). Two SNaPshot® SBE Multiplex systems were specifically designed for this study, successfully optimized and used for genotyping. A total of 20 *SLC22A2* gene 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 M1V; R176H; C282G; L351W; R207H; T357M; M393T; R404C; G439E; R463K; R487Q R487W; V502E/G; and V502M 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.

4.3.3. DNA isolation and SNaPshot™ genotyping

DNA isolation and SNaPshot™ genotyping were performed as described in **Chapter 2**.

4.3.4. Electrophoresis of the minisequencing products

The purified fluorescently labelled minisequencing products were mixed with HiDi™ formamide and GeneScan-120 Liz size standard (Applied Biosystems), denatured, and separated on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems) as described in **Chapter 2**. Data analyses were performed using GeneMapper® IDX Software Version 1.2.

Table 4.1 - 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 4.2 - *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)	polyGACT tail
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

Table 4.3 - *SLC22A2* gene multiplex 2 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)	polyGACT tail
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

4.4. Results

4.4.1. SNaPshot™ Genotyping

To determine the allele frequencies of 20 known coding variants of OCT2 we screened 148 Xhosa individuals. The genotype and allele frequencies of the 20 SNPs are summarized in **Table 4.5.** A typical electropherogram of the SLC22A2 SNaPshot genotyping system is displayed in **Figure 4.1**. All the variants observed were in Hardy-Weinberg equilibrium ($p>0.05$). Seventeen out of the 20 investigated nonsynonymous SNPs were monomorphic in the Xhosa population. Heterozygosity was only observed for three of the investigated nonsynonymous SNP in this study as shown in Table X. The A270S (rs316019) genotype frequencies for wild-type (GG), heterozygote (GT), and homozygote (TT) were 87.8%; 12.2%; and 0.0% respectively. The MAF for A270S (rs316019) observed in this study was 6.1%. The genotype frequencies for the R400C (rs8177516) variant wild-type (CC), heterozygote (CT), and homozygote (TT) were 93.2%; 6.8%; and 0.0%, respectively. The MAF for R400C (rs8177516) was 3.4%. The K432Q (rs8177517) genotype frequencies wild-type (AA), heterozygote (AC), and homozygote (CC) were 98.6%; 1.4%; and 0.0%, respectively. The MAF for the K432Q (rs8177517) was 0.7%.

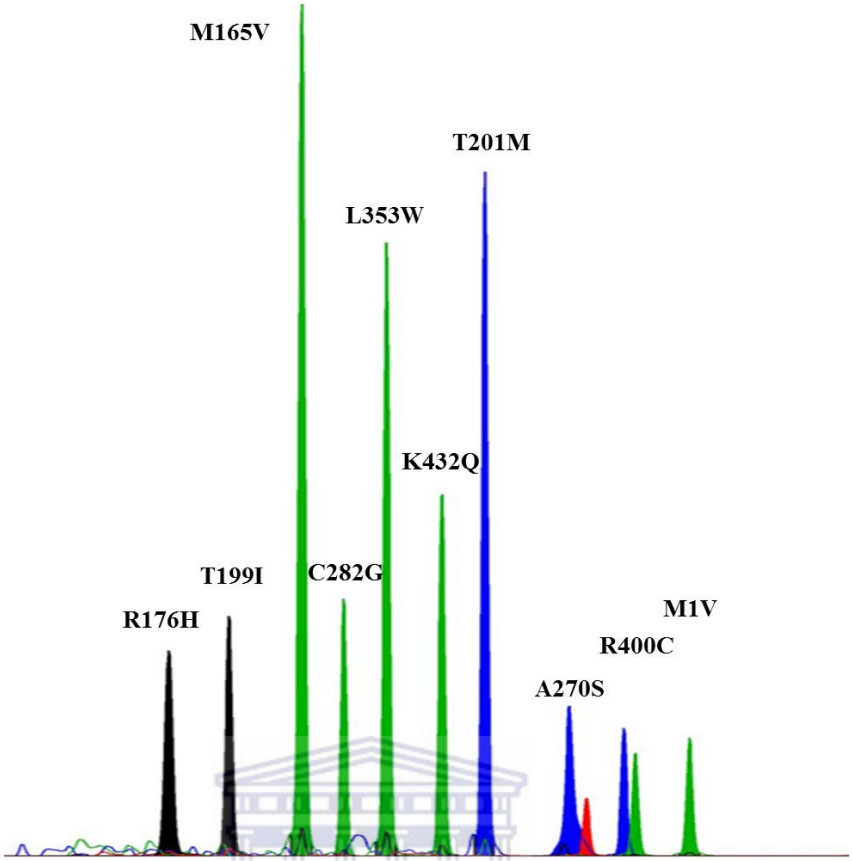

Figure 4.1 – Typical electropherogram of *SLC22A2* multiplex 1 minisequencing fragments. Black represents C, green A, blue G, and red T.

Table 4.4 – Haplotype structure of *SLC22A2* gene in the Xhosa population as defined by the 20 selected loci.

Haplotype Id	Haplotype	Frequency
Haplotype *1	AAGGCGGTTGACGAGCCGAC	90.0%
Haplotype *2	AAGGCGTTTGACGAGCCGAC	6.0%
Haplotype *3	AAGGCGGTTGATGAGCCGAC	3.2%
Haplotype *4	AAGGCGGTTGACGCGCCGAC	0.7%
Haplotype *5	AAGGCGTTTGATGAGCCGAC	0.1%

4.4.2. Haplotype Analysis

Haplotypes were constructed using the SHesis online platform. Five haplotypes listed in **Table 4.4** were identified from the 20 investigated nonsynonymous SNPs. The most observed haplotype was Haplotype *1 which had a frequency of 90.0%. Haplotype *2 and *3 were observed at frequencies of 6.0% and 3.2%, respectively. The two least observed haplotype, Haplotype *4 and *5, were observed at frequencies of 0.7% and 0.1%, respectively.

Table 4.5 - Genotype and allele frequencies of 20 known *SLC22A2* coding SNPs in 148 healthy Xhosa individuals.

Amino Acid Substitution	dbSNP ID	Observed Genotype Frequency			Allele Frequency			
		Genotype	%	95% CI	Allele	%	95% CI	HWE (P)
M1V	rs141582772	CC CT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C T	98.3 1.7	98.4 – 100.0 0.0 – 1.6	
M165V	rs8177508	TT TC CC	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	T C	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
R176H	rs57371881	TT TA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	T A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
T199I	rs201919874	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	
T201M	rs145450955	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	
R207H	rs199783132	CC CT TT	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	C	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
S270A	rs316019	TT TG GG	87.8 12.2 0.0	81.5 – 92.2 7.2 – 17.8 0.0 – 1.9	C T	91.6 8.4	90.9 – 96.4 3.6 -9.1	0.655
C282G	rs144729356	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	
L351W	rs45599131	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	

T357M	rs184227446	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	
M393T	rs141205337	GG GT TT	100.0 0.0 0.0	88.6 – 96.9 0.0 – 1.3 0.0 – 1.3	G T	100.0 3.0	98.4 – 100.0 0.0 – 1.6	
R400C	rs8177516	CC CT TT	91.3 8.5 0.0	87.8 96.4 3.1 – 11.4 0.0 – 1.9	A C	96.6 3.4	94.2 – 98.4 18.2 – 23.0	0.886
R404C	rs137885730	CC CG GG	100.0 0.0 0.0	87.8– 96.4 0.0 – 1.3 0.0 – 1.3	C G	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
K432Q	rs8177517	AA AC CC	98.6.0 1.4 0.0	94.8 – 99.9 0.0 – 4.2 0.0 – 1.9	A G	100.0 0.0	97.9 – 100.0 0.0 – 2.1	0.934
G439E	rs141405449	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	
R463K	rs3907239	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	
R487Q	rs151282335	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	
R487W	rs140829992	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	
V502E/G	rs17853948	TT TA AA	100.0 0.0 0.0	96.9 – 100.0 0.0 – 1.3 0.0 – 1.3	T A	100.0 0.0	98.4 – 100.0 0.0 – 1.6	
V502M	rs140033522	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	

Table 4.6 - Comparison of MAF of selected *SLC22A2* SNPs in the Xhosa population to other ethnic groups.

dbSNP ID	Amino acid change	Minor Allele	Minor Allele Frequency (%)							
			Xhosa[a]	Luhya[b]	Yoruba[b]	African-American[b]	Japanese[b]	Chinese-Han[b]	Caucasian-Finish[b]	Caucasian-American[b]
rs8177508	M165V	T	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0
rs201919874	T199I	T	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	T	8.4	16.0	15.9	14.8	0.0	14.7	5.9	8.8
rs8177516	R400C	T	3.4	0.0	2.3	1.6	0.0	0.0	0.0	0.0
rs8177517	K432Q	T	1.4	5.2	6.2	1.6	0.0	0.0	0.0	0.0

a.This study; b. Data from 1000 Genomes

4.5. Discussion

The kidney-specific hOCT2 is responsible for therapeutic drug uptake from the circulation into renal proximal tubules. Therefore it is an important determinant of drug disposition and response, and adverse events that result from drug-drug interactions. Genetic polymorphisms of the *SLC22A2*, which codes for hOCT2, have been implicated in altered transport which in turn influences drug disposition and response. However, little information is available on the genetic variation of *SLC22A2* in African populations residing in southern Africa. Therefore, the aim of this study was to develop a SNaPshot genotyping system in order to assess the baseline allele frequency distributions of previously identified nonsynonymous *SLC22A2* SNPs in the Xhosa population of South Africa.

The reduced-function variant M165I (rs8177508) was first observed by Leabman *et al.* (2002) in African-American participants who formed part of a group of 247 ethnically diverse individuals (Leabman *et al.*, 2002). The minor allele frequency recorded for the M165I variant in the Leabman study was 1%. However, this variant was not observed in the current study and is also absent from those African populations included in the 1000 Genomes database.

The two nonsynonymous *SLC22A2* variants T199I (rs201919874) and T201M (rs145450955) were previously identified as rare SNPs in Asian populations with MAF <1% ((Fukushima-Uesaka *et al.*, 2004, Kang *et al.*, 2007). These variants showed decreased transport of prototypical organic cations in functional studies *in vitro* which were not attributable to either protein expression or plasma membrane localization. The T199I (rs201919874) and T201M (rs145450955) were not

observed in this study and can be considered an Asian-specific variants since it has not been observed in any of the other population groups.

The A270S (rs316019) variant has been identified as a common SNP that occurs at a high allelic frequency (7-15%) in all ethnic groups studied (Fujita *et al.*, 2006, Kang *et al.*, 2007, Leabman *et al.*, 2002). The MAF for the A270S (rs316019) variant in the Xhosa population was recorded at 6.1% which is significantly lower than the global MAF for this variant of 12%. Moreover, the MAF for the Xhosa population was substantially lower than that recorded for the Luhya of Kenya (16%), and Yoruba of Nigeria (15.9%), showing that inter-ethnic variability does exist between populations in Southern Africa and those from West and East Africa for the A270S (rs316019) variant. The functional activity of the A270S (rs316019) variant has been well characterized, and it has been shown to result in decreased transport of prototypical substrates *in vitro*, compared with the wild-type. Recent studies by Song *et al.* (2008) and Wang *et al.* (2008) showed significantly lower renal clearance of metformin in carriers homozygous for A270 (rs316019)S as compared to homozygous wild-type (Song *et al.*, 2008a, Wang *et al.*, 2008). This observation was supported by Tzvetkov *et al.* (2009) who made a similar in a population of Caucasian men (Tzvetkov *et al.*, 2009). In addition, Li *et al.* (2010) showed that the A270S (rs316019) variant can affect the plasma lactate level and the incidence of hyperlactacidemia in type-2 diabetes patients on metformin therapy (Li *et al.*, 2010).

The R400C (rs8177516) variant of hOCT2 is of particular interest because it changes an arginine that is evolutionary conserved amongst the OCT paralogs (OCT1-3) (Leabman *et al.*, 2002). The MAF for R400C (rs8177516) in this study was 6.8%, compared to 2.3% and 1.6% MAF reported for the Yoruba and African Americans in

the 1000 Genomes project, respectively. This variant has thus far only been observed in African populations (Fukushima-Uesaka *et al.*, 2004, Kang *et al.*, 2007, Leabman *et al.*, 2002). Functional analysis of R400C (rs8177516) revealed that this variant had significant reduced transport activity of prototypical cationic substrates *in vitro* compared to the hOCT2 reference (Leabman *et al.*, 2002). However, the role of this variant on the pharmacokinetics and efficacy of its substrate drugs *in vivo* had not been establish yet. Given the high MAF of this variant in the Xhosa individuals studied, and its effect on hOCT2 transport activity *in vitro*, the impact of this variant on drug disposition and efficacy in the Xhosa population requires closer investigation.

Another hOCT2 variant, K432Q (rs8177517) , in an *in vitro* assay showed a stronger affinity for the prototypical organic cation MPP^+ and a sensitivity to TBA (Leabman *et al.*, 2002). This variant has thus far only been observed in Admixed Americans, Colombians, African Americans, and Africans. The MAF for K432Q (rs8177517) observed in this study was 1.4%, compared to that observed in the 1000 Genomes project of 6.6%, 5.6%, and 1.6% for the Luhya, Yoruba and African Americans, respectively. Although this variant has shown reduced transport activity *in vitro*, no information is available on the clinical impact of this SNP *in vivo*. Thus, given the fact that this variant occurs at a higher allele frequency in African populations, the *in vivo* effect of this variant on hOCT2 substrate drug transport requires further scrutiny.

4.6. Conclusions

In this study the 20 investigated nonsynonymous *SLC22A2* SNPs showed a low level of heterozygosity, with 85% of the loci displaying monomophism in the Xhosa subjects. This study once again highlighted the fact that the distribution of SNPs and the MAFs thereof differ between African 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 inter-ethnic and inter-individual variability in drug response for substrate drugs of hOCT2. Moreover, the single SNPs identified and haplotypes inferred for the Xhosa population may be important in future pharmacogenetic in identifying association between causative variants and altered drug response. While **Chapters 1-3** have dealt with genetic variation in the uptake and efflux transporters which are co-localized in the liver and kidney, respectively, **Chapter 5** explores genetic variations of *SLC22A3*, the gene of the ubiquitously expressed organic cation transporter hOCT3

CHAPTER 5

Evaluation of 18 Single Nucleotide Polymorphisms in the *SLC22A3* Gene of the Xhosa Population

5.1. Abstract

Organic cation transporters of the amphiphilic solute facilitator family of membrane proteins are involved in the translocation of a diverse range of endogenous and exogenous organic cations. These organic cations include clinically important substrates such as the anti-diabetic drug metformin, the anti-neoplastic drug oxaliplatin, and the anti-retroviral drug lamivudine. Inter-individual variation in drug disposition and efficacy is a major clinical problem and the role of these transporters is increasingly being recognized as a possible mechanism explaining this variation. Moreover, it is estimated that genetic factors can account for between 15-30% of inter-individual variation of drug disposition and response. The aim of this study was to determine the baseline minor allele frequency distribution of 18 known coding SNPs in the *SLC22A3* gene of 148 Xhosa individuals residing in Cape Town, South Africa. This study found no genetic polymorphisms in the coding region of the *SLC22A3* gene of the Xhosa individuals investigated. To our knowledge this study represents the first of its kind to investigate the baseline allele and genotype frequency distributions of known genetic polymorphisms within the *SLC22A3* gene of the Xhosa population. This study has shown that *SLC22A3* coding SNPs observed in other populations are absent in the sample of Xhosa individuals studied. The lack of protein sequence variation was consistent with other studies and may reflect the significant physiological role of hOCT3 in maintaining cellular and organismal homeostasis.

5.2. Introduction

Organic cation transportes (OCTs) belong to the amphiphilic solute facilitator (ASF) family integral transmembrane proteins and are involved in various metabolic processes and detoxification (Schömig *et al.,* 1998). These transporters are characterized by a specific organ and species-dependent expression and mediate the transport of organic cations (OCs) in an electrogenic and Na^+-independent manner (Burckhardt and Wolff, 2000).

The human organic cation transporter 3 (hOCT3), also known as extraneuronal 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 polyspecific transporter that is involved in the cellular uptake and elimination of small OCs with different molecular structures. These OC substrates include endogenous bioamines, 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 1-methyl-4-pyridinium (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.*, 1997, Verhaagh *et al.,* 1999). The *SLC22A3* gene is 77kb in length and consists of 11 exons with consensus GT/AG splice sites and conserved intron locations (Gründemann and Schömig, 2000). Moreover, the *SLC22A3* gene contains two transcriptional start points and the promoter, located

within a CpG island, lacks a canonical TATA box but contains a prototypical initiator element and a number of potential binding sites for ubiquitous transcription factors SpI and NF-1 (Gründemann and Schömig, 2000). Recently, Chen *et al.* (2013) demonstrated that genetic polymorphisms in the proximal promoter region of *SLC22A3* alter the transcription rate of the gene and may be associated with altered expression levels of hOCT3 in the liver (Chen *et al.*, 2013). In addition, they also showed that hypermethylation of the CpG island in the proximal promoter region is the probable mechanism accounting for decreased expression of hOCT3 in prostate cancer.

The ubiquitously expressed hOCT3 is also increasingly been recognized as an important transporter of anti-cancer drugs. For example, a study by Yokoo *et al.* (2008) investigated whether hOCT3 was significantly involved in oxaliplatin-induced cytotoxicity and accumulation of platinum in colorectal cancer (Yokoo *et al.*, 2008). They concluded that hOCT3-mediated uptake of oxaliplatin into cancer cells was indeed important for its toxicity, and that hOCT3 may be a marker for cancer chemotherapy. In another study, Shnitsar *et al.* (2009) found that renal cell carcinoma (RCC) cell lines, usually chemoresistant, expressing hOCT3 increases chemosensitivity to the antineoplastics, melphalan, irinotecan, and vincristine (Shnitsar *et al.*, 2009). In a recent study, Li *et al.* (2012) found that hOCT3 also partially contributed to the sensitivity of human cervical adenocarcinoma cells to cisplatin cytotoxicity (Li *et al.*, 2012).

The hOCT3 gene, *SLC22A3*, was also identified as an important risk locus for prostate cancer, and was markedly under-expressed in aggressive prostate cancers (Eeles *et al.*, 2008). This study also revealed that hypermethylation of the *SLC22A3*

promoter region in prostate cancer was one of the important mechanisms for the reduced expression of this transporter. Furthermore, a study by Mohelnikova-Duchonova *et al.* (2013) found a significant upregulation of *SLC22A3* in pancreatic ductal adenocarcinoma (PDAC) tumours compared to non-neoplastic tissues (Mohelnikova-Duchonova *et al.*, 2013). In addition to cancer, the *SLC22A3-LPAL2-LPA* gene cluster was also previously identified in a genome-wide association (GWAS) haplotype study as a risk locus for coronary artery disease (CAD) (Trégouët *et al.*, 2009).

The biguanide antidiabetic drug metformin is usually the first-line therapeutic used in the treatment of type-2 diabetes (Kirpichnikov *et al.*, 2002, Nathan *et al.*, 2009). The action of metformin appears to be related to its activation (phosphorylation) of the energy sensor AMP-activated kinase (AMPK), which results in suppression glucagon-stimulated glucose production and enhancement of glucose uptake in muscle and hepatic cells (Abbud *et al.*, 2000, Zhou *et al.*, 2001). Previous studies have shown that OCTs, the hOCT3 paralogues hOCT1 and hOCT2, together with MATEs play a critical role in the disposition response and that genetic variants of these transporters are associated with variation in pharmacokinetic and anti-diabetic action of the drug (ML Becker *et al.*, 2009, Becker *et al.*, 2010, Kimura *et al.*, 2009, Yan Shu *et al.*, 2007, Shu *et al.*, 2008). Subsequently, a study by Chen *et al.* (2010) has suggested that in addition to hOCT1, hOCT2 and MATE1, hOCT3 should be considered an important mechanism for metformin uptake in muscle cell types and that variation in this transporter may modulate the response to metformin (Chen *et al.*, 2010a).

The role of hOCT3 is not only recognized as a pharmacogene in the transport of metformin but is also considered a promising drug target in antidepressant therapy (Zhu *et al.*, 2012). Because of its expression pattern and physiological profile, hOCT3 is also considered as a candidate gene for various neuropsychiatric disorders of inherent monoaminergic dysfunction (Wultsch *et al.*, 2009). hOCT3's importance in the regulation of neurotransmission has been well documented in a number of animal studies (Amphoux *et al.*, 2006, Baganz *et al.*, 2008, Cui *et al.*, 2009, Wultsch *et al.*, 2009) and more recently, in humans (Cui *et al.*, 2009). Aoyama *et al.* (2006) found that certain SNPs in the *SLC22A3* gene were associated with the development of polysubstance abuse in Japanese individuals with dependence on the amphetamine derivative methamphetamine (Aoyama *et al.*, 2006). In addition, Lazar et al. (2008) in a case-control study of 84 Caucasian children and adolescents found that known *SLC22A3* genetic polymorphisms were not associated with obsessive compulsive disorder (OCD) in their study sample, but did identify two novel polymorphisms that were associated with OCD (Lazar *et al.*, 2008).

Mental disorders are a major contributor to the burden of disease globally, with about 14% of the global burden being attributed to neuropsychiatric disorders (Prince *et al.*, 2007, Tomlinson *et al.*, 2009). Moreover, in sub-Saharan Africa neuropsychiatric disorders account for nearly 10% of the total burden of disease (Lopez *et al.*, 2006, Tomlinson *et al.*, 2009). The genetic basis for several diseases, including neuropsychiatric disorders is well established. To bridge the gap in pharmacogenetic mapping in African populations, especially those residing in southern Africa, this study prioritized the genotyping of 18 known variable sites in the coding region of the *SLC22A3* gene in the Xhosa population living in the Cape Town, South Africa.

5.3. Materials and Methods

5.3.1. Subjects

As described in **Chapter 2**.

5.3.2. DNA extraction and SNP selection

Isolation of genomic DNA from buccal swab samples was carried out as outlined in **Chapter 2** using a standard salt-lysis protocol and stored frozen at -20°C until the time of genotyping (Leat *et al.*, 2004a). A total of 18 *SLC22A3* 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 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.

5.3.3. Primer design

Multiplex PCR primers for the amplification of all 11 *SLC22A3* exons and flanking regions were designed using Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3) and are listed in **Table 5.1**. To test for possible non-specific amplification, primers were aligned with the NCBI sequence databases using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast/blast-cgi). 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 5.2** and **5.3**.

Table 5.1 - 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

5.3.4. Multiplex PCR

All the 11 *SLC22A3* exons and their flanking regions were simultaneously amplified using the primers listed in **Table 5.1**. The PCR reactions were performed in a 20 μl volume, containing 20 – 50 ng of genomic DNA, 1 x Qaigen multiplex PCR master mix (Qaigen, Courtaboeuf, France) and 0.2 μM of each primer. Cycling parameters used and PCR product purification were as described in **Chapter 2**.

5.3.5. Multiplex minisequencing reactions

Multiplex minisequencing was performed in a 10 μl reaction volume using 3 μl of a 1/10 dilution of purified PCR products, 0.1-0.2 μM of primers, and 5 μl of SNaPshot™ ready reaction mix (Applied Biosystems). Sequence cycling and post-extension treatment was performed according to the instructions of the manufactureras described in **Chapter 2**

5.3.6. Electrophoresis of the minisequencing products

The fluorescently labelled fragments were separated on 36 cm-long capillaries in POP4 polymer on an ABI Prism 3500 Genetic Analyzer (Applied Biosystems) as described in **Chapter 2**.. Data analyses were performed using GeneMapper® IDX Software Version 1.2.

Table 5.2 - *SLC22A3* gene multiplex 1 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)	polyGACT tail
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

Table 5.3 - *SLC22A3* gene multiplex 2 single base extension primers for SNaPshot™ genotyping of selected SNPs.

NCBI (dbSNP)	Amino Acid Change	Nucleotide change	Nucleotide sequence (5′ → 3′)	Position Accession number (NC_000006.12)	Primer length (bp)	polyGACT tail
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

5.4. Results and Discussion

The population studied consisted of 148 healthy Xhosa individuals between the ages of 18 and 61 years. There were 80 (54%) female and 68 (46%) 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.

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 148 Xhosa subjects are summarized in **Table 5.4**. All 18 coding SNPs genotyped in this study were monomorphic in the Xhosa population.

Table 5.4 - Genotype and allele frequencies of the OCT3 *(SLC22A3)* gene SNPs in 148 healthy Xhosa individuals.

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

Over the last number of years hOCT3 has increasingly being recognized as an anti-diabetic and anti-cancer drug transporter (Chen *et al.*, 2010a, Nies *et al.*, 2009, Yokoo *et al.*, 2008). Recently published reports also provide evidence of the increased interest in the role of hOCT3 in neurotransmission and maintenance of homeostasis in the central nervous system (CNS) as a result of its recognized ability to translocate monoamines (Amphoux *et al.*, 2006). In addition, hOCT3 is also drawing interest as a potential target in the treatment of selected neuropsychiatric disorders.

In the current study we genotyped 18 known SNPs in the *SLC22A3* gene of 152 Xhosa 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. However, this lack of genetic variation in the coding region of *SLC22A3* in the Xhosa population is not a unique situation and has also been observed in other populations (Kang *et al.*, 2007, 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 psycho-stimulant 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 anti-cancer drugs, but more recently also as a biomarker for cancer pathogenesis. For example, in colorectal cancers hOCT 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 anti-diabetic drug and is widely used as a first-line therapeutic in the treatment of type-2 diabetes. Earlier studies have shown that metformin is transported by OCT1 and OCT2, and that genetic polymorphisms of these transporters affect the pharmacokinetic and therapeutic effect of the drug (ML Becker *et al.*, 2009, Becker *et al.*, 2010, Kimura *et al.*, 2009, Yan 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 OCT3 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 type-2 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 Xhosa 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 Xhosa 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 non-coding elements (Ward and Kellis, 2012). However, the genotyping strategy adopted in this study have excluded the typing of non-coding SNPs, which could be useful when performing linkage disequilibrium analysis or extracting information about disease association.

5.5. Conclusions

To our knowledge this study represents the first of its kind to investigate the baseline allele and genotype frequency distributions of known genetic polymorphisms within the *SLC22A3* gene of the Xhosa population. This study has shown that *SLC22A3* coding SNPs observed in other populations are absent in the sample of Xhosa individuals studied. The lack of protein sequence variation was consistent with other studies and may reflect the significant physiological role of hOCT3 in maintaining cellular and organismal homeostasis. The lack of heterozygosity at known polymorphic sites observed within *SLC22A1*-3 and *SLC47A1* in the Xhosa subjects prompted the question whether the Xhosa subjects harboured any novel variants within these genes. Since kidney-specific hOCT2 is a critical determinant of drug disposition and toxicity, and a known site of drug-drug interactions, **Chapter 6** prioritized the sequencing of the *SLC22A2* gene to search for novel genetic variants within the Xhosa population.

CHAPTER 6

Identification of Novel Genetic Variations within the *SLC22A2* Gene of the Xhosa Population

6.1. Abstract

Human organic cation 2 (hOCT2) is an important determinant of organic cation uptake from the circulation into the renal proximal tubules. These organic cations include clinical drugs such as metformin, lamivudine, cimetidine, cisplatin and the neurotoxin 1-methyl-4-phenyl-pyridinium. Considerable interindividual variation exists in drug responses and toxicity. 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 pharmacogenetically relevant organic cation transporter (OCT) genes of indigenous southern African populations. Therefore, the aim of this study was to determine whether the *SLC22A2* gene of the Xhosa participants harbours any novel SNPs using direct sequencing of the 11 exonic and flanking intronic regions of the gene in 96 of the participants. Twenty-three genetic polymorphisms, including 7 novel SNPs, were identified in the *SLC22A2* gene of the Xhosa individuals that participated in this study. This study represents the first report of novel *SLC22A2* SNPs in the Xhosa population. The rare singleton SNPs identified and haplotypes inferred for the Xhosa population in this study is an important step in filling the gap with regards to genetic information on the pharmacogenetically relevant *SLC22A2* gene in indigenous southern African populations. The information generated in this study can potentially

lay the foundation for future pharmacogenetic study design and for the identification of association between the rare SNPs and drug response and toxicity.

6.2. Introduction

Human organic cation 2 (hOCT2) is primarily expressed in the kidney and located on the basolateral membrane of renal proximal tubules (Gorboulev *et al.*, 1997, Motohashi *et al.*, 2002). Because of this tissue distribution and membrane localization hOCT2 is thought to play a critical role in the uptake, pharmacological effects and/or adverse effects of many cationic clinical therapeutics and xenobiotics. Examples of clinical drugs transported by hOCT2 include metformin (antidiabetic), lamivudine (antiretroviral), cisplatin (antineoplastic) and cimetidine (antihistamine) (Jung *et al.*, 2008, Ciarimboli *et al.*, 2005b, Kimura *et al.*, 2005b, Koepsell *et al.*, 2007). In addition, hOCT2 is also involved in the translocation of endogenous bioactive amines such as dopamine and norepinephrine, and in the elimination of toxic substances such as the neurotoxin 1-methyl-4-phenyl-pyridinium (MPP^+) (Burckhardt and Wolff, 2000, Dresser *et al.*, 2002, Gorboulev *et al.*, 1997, Okuda *et al.*, 1999). Clinical studies and *in vivo* animal experiments with knockout mice have demonstrated that variation in the expression level of *SLC22A2* can be responsible for individual variation in pharmacokinetics. Moreover, *SLC22A2* genetic polymorphisms have been implicated in the altered function of hOCT2 which may lead to a change in the disposition and response of substrate drugs.

To date several single nucleotide polymorphisms (SNPs) have been identified in the *SLC22A2* gene of ethnically diverse populations (Fukushima-Uesaka *et al.*, 2004, Kang *et al.*, 2007, Leabman *et al.*, 2002, Tzvetkov *et al.*, 2009). Functional

characterization have revealed that several of these SNPs affect the transport function of hOCT2 *in vitro* (Leabman *et al.*, 2002). Although *in vivo* evidence for the involvement of these SNPs in clinical phenotype is limited, 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 wild-type (Song *et al.*, 2008a, Wang *et al.*, 2008). Furthermore, this reduced-function *SLC22A2* SNP, rs316019, was also associated with reduced nephrotoxicity from cisplatin in cancer patients (Filipski *et al.*, 2009). However, these pharmacogenetic 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 pharmacogenetic genes can differ significantly between populations and even within populations (Drögemöller *et al.*, 2010, Yen-Revollo *et al.*, 2009). In addition, population-specific variants exist in non-Caucasians which will probably be more relevant 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 HapMap 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. Thus, in order to fully understand and correlate this genomic diversity with pharmacogenetic phenotypes, the extent of variation in pharmacogenetically relevant genes such as *SLC22A2* in more African populations needs to be studied. Therefore, the aim of this study was to determine whether the *SLC22A2* gene of the Xhosa participants harbours any novel SNPs, using direct sequencing of the 11 exons and flanking intronic regions of the gene in 96 healthy individuals. Secondly, to determine the haplotype structure of the *SLC22A2* gene based on the genetic information acquired by sequencing, and finally, to compare the minor allele frequencies obtained for the Xhosa to the HapMap estimates for other African, American, European and Asian populations.

6.3. Materials and Methods

6.3.1. Subjects

The DNA from 96 of the 148 unrelated healthy Xhosa subjects (As described in **Chapter 2**) was used to screen for novel SNPs by direct sequencing.

6.3.2. 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.*, 2004a). The proximal promoter region and 11 exonic fragments of the *SLC22A2* gene were generated using self-designed primer sequences. These primers were

designed using Primer3 software (www.genome.wi.mit.edu/cgi-bin/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 Qaigen multiplex TopTaq master mix (Qaigen, 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. The fragments were sequenced at Macrogen (Seoul, South Korea).

6.3.3. Statistical Analysis

Genotype and allele frequencies as well as the deviation from the Hardy-Weinberg Equilibrium were calculated using as described in Chapter 2. Statistical significance was defined as $p<0.05$.

Table 6.1 - PCR primer sequences for *SLC22A2* sequencing amplicon generation.

Location	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Amplified region (NC_000006.12)	Amplicon length (bp)
Promoter	GGGAAGATTACTGGGCTGTG	GAGAGCAGAGCCAAGAGGAA	160259130 - 160258669	462
Exon 1	GCTGGTCACTTGCAGAGGTA	TCTCCACCATTTGCTTCTCC	160258886 - 160258297	590
Exon 2	AGGGCAAGCCTTTTGGTTAT	GAAAGGATGGGATTCAAGCA	160257099 - 160256522	578
Exon 3	GGGTATTCAGCACAGGATGG	GAAGCTGGGTCCCTTTTCTT	160251022 - 160250450	573
Exon 4	AGCTGGACAGCCAACTCATT	TTCCTCTGAGTGGGGAGAGA	160249445 - 160248858	588
Exon 5	ATCCAGTCCTTGACCCCTCT	CTCTGTTGCATTCCGCTACA	160247625 - 160247038	588
Exon 6	ATTGCACCACTGCACTCAAG	GGGGTTTTGGCTTTGGTATT	160245764 - 160245169	596
Exon 7	CACAGCCAGCCACTGAAGTA	GCTGGCCATATGAATTTGCT	160243961 - 160243408	554
Exon 8	CCTTCCTCTCCATTTTGCTG	TTGGGTAATCCCTGTCTTGC	160242666 - 160242059	608
Exon 9	GTTTATTCAGGGGTGGATGG	TCAGGAAGGGTGGAAATCAG	160241663 - 160241110	554
Exon 10	CAGCAGTCAGAGATGGCAAA	TTGTTAGGAAAATTAGCCCAATG	160224947 - 160224328	620
Exon 11	TGGCATTCACGAAGACAAGA	GCTGCCATCAAAGCTAGGTC	160217981 - 160217387	595

6.4. Results

6.4.1. Variant Screening

To identify variants of hOCT2 we screened all 11 exons and the proximal promoter region of the *SLC22A2* gene in 96 Xhosa participants, using direct sequencing. Twenty-eight variable sites including four novel ones were found in this study **(Table 6.2)**. Eight of these variations were in the coding region and 20 were in the non-coding or intronic region of the gene. Four of the SNPs present in the coding region were non-synonymous and four were synonymous substitutions.

The four non-synonymous SNPs observed in this study, A270S (rs316019), R400C (rs8177516), K432Q (rs8177517), and N552I (rs139045661) had ethnic-specific minor allele frequencies (MAFs) greater than or equal to 1%. The R400C (rs8177516) and N552I (rs139045661) variants have thus far only been observed in African populations or ethnic groups with a link to the African continent such as the African-Americans. Two of the synonymous SNPs, T130T (rs624249) and V502V (rs316003) were reported previously by Leabman *et al*. (2002) and Fukushima *et al*. (2004), while the S133S (rs112210325) variant was only submitted to the dbSNP database in the last year and had not been reported in any study yet. In addition, a novel synonymous SNP V94V (MBPG_OCT2002) in exon 1 **(Figure 6.1)** had been identified in this study and will be submitted to the dbSNP database in due course.

Table 6.2 - Summary of SNPs identified with direct sequencing in the *SLC22A2* gene of Xhosa subjects.

dbSNP ID	Location	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′)	Amino Acid Position	Amino Acid change	MAF	HWE
rs55920607	Promoter	160259003	-246	C>T			0.084	0.370
rs59695691	Promoter	160258952	-195	A>G			0.263	0.000
MBPG_OCT2001	Promoter	160258913	-156	C>T			0.005	0.959
rs150063153	Promoter	160258852	-95	A>C			0.016	0.876
MBPG_OCT2002	Exon 1	160258476	282	G>A	94	Val>Val	0.011	0.914
rs624249	Exon 1	160258368	390	G>T	130	Thr>Thr	0.128	0.566
rs112210325	Exon 1	160258359	399	G>T	133	Ser>Ser	0.012	0.913
rs8177511	Intron 2-3	160250720	-18	T>C			0.026	0.793
rs112710522	Intron 3-4	160250473	+75	A>G			0.086	0.581
rs372467753	Intron 4-5	160247329	-31	A>G			0.011	0.958
rs316019	Exon 4	160249250	808	T>G	270	Ser>Ala	0.149	0.090
rs112425400	Intron 4-5	160247418	-120	G>A			0.011	0.917
rs2279463	Intron 4-5	160247357	-59	T>C			0.183	0,536
MBPG_OCT2003	Intron 6-7	160245318	+65	G>T			0.010	0.918

Table 6.2 Continued - Summary of SNPs identified with direct sequencing in the *SLC22A2* gene of Xhosa subjects.

rs617217	Intron 6-7	160245324	+71	G>C			0.278	0.661
rs115889347	Intron 6-7	160245346	+93	G>A			0.005	0.959
MBPG_OCT2004	Intron 6-7	160245368	+115	G>C			0.052	0.590
rs8177516	Exon 7	160243653	1198	C>T	400	Arg>Cys	0.052	0.590
rs8177517	Exon 8	160242388	1293	A>C	432	Lys>Gln	0.011	0.917
rs17588242	Intron 8-9	160242198	+96	A>G			0.011	0.917
rs11967308	Intron 9-10	160241327	+147	G>A			0.146	0.973
rs114897022	Intron 9-10	160241316	+158	A>G			0.016	0.876
MBPG_OCT2005	Intron 9-10	160241261	+213	A>T			0.005	0.959
MBPG_OCT2006	Intron 9-10	160241210	+272	G>A			0.005	0.959
rs316003	Exon 10	160224800	1506	G>A	502	Val>Val	0.333	0.635
MBPG_OCT2007	Intron 10-11	160217752	-254	C>A			0.005	0.959
rs3103352	Intron 10-11	160217693	-195	C>T			0.235	0.00015
rs139045661	Exon 11	160217445	1656	T>A	552	Ile/Asn	0.016	0.876

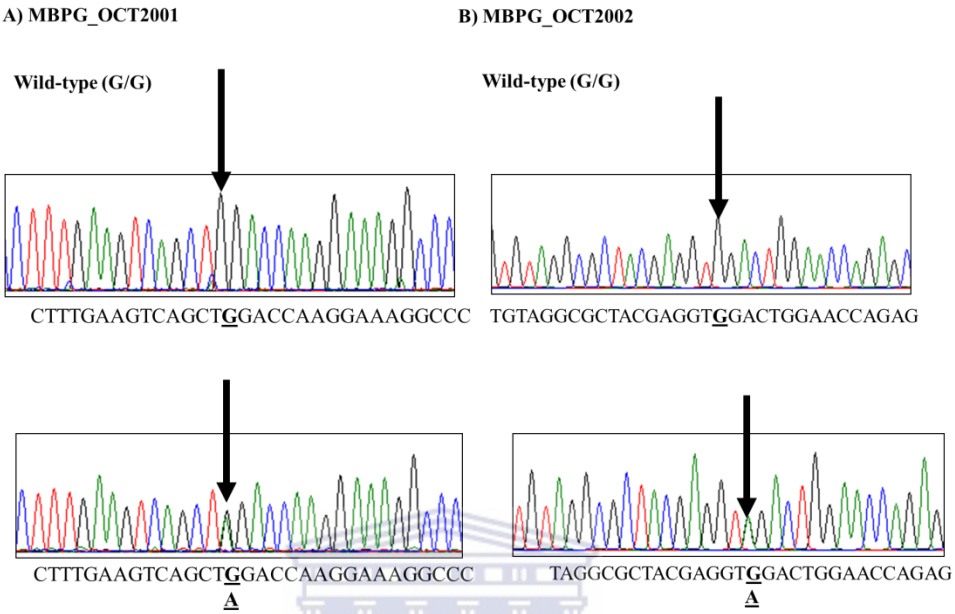

Figure 6.1 - Electropherograms of novel SNPs observed in the proximal promoter region and exon 1 of the *SLC22A2* gene. (A) MBPG_OCT2001 (wild-type -156 G/G and variant -156 G/A) a novel SNP detected in the proximal promoter region. (B) MBPG_OCT2002 (wild-type 282 GG; variant 282 G/A) a novel synonymous SNP detected in exon 1.

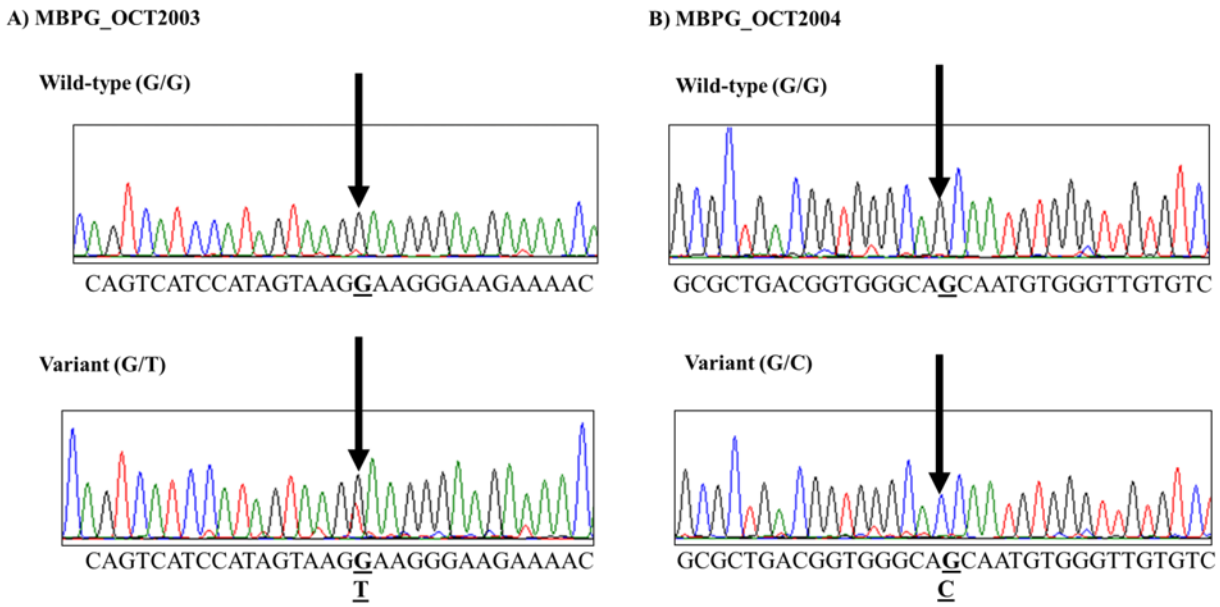

Figure 6.2 - Electrophoretograms of novel SNPs observed in the intronic region between exon 6 and 7 of *SLC22A2*. (A) MBPG_OCT2003 (wild-type MBPG_OCT2003G/G and variant MBPG_OCT2003G/T) a novel SNP detected in the intronic region between exons 6 and 7. (B) MBPG_OCT2004 (wild-type MBPG_OCT2004 G/G; variant MBPG_OCT2004G/C) a novel SNP detected in the intronic region between exons 6 and 7.

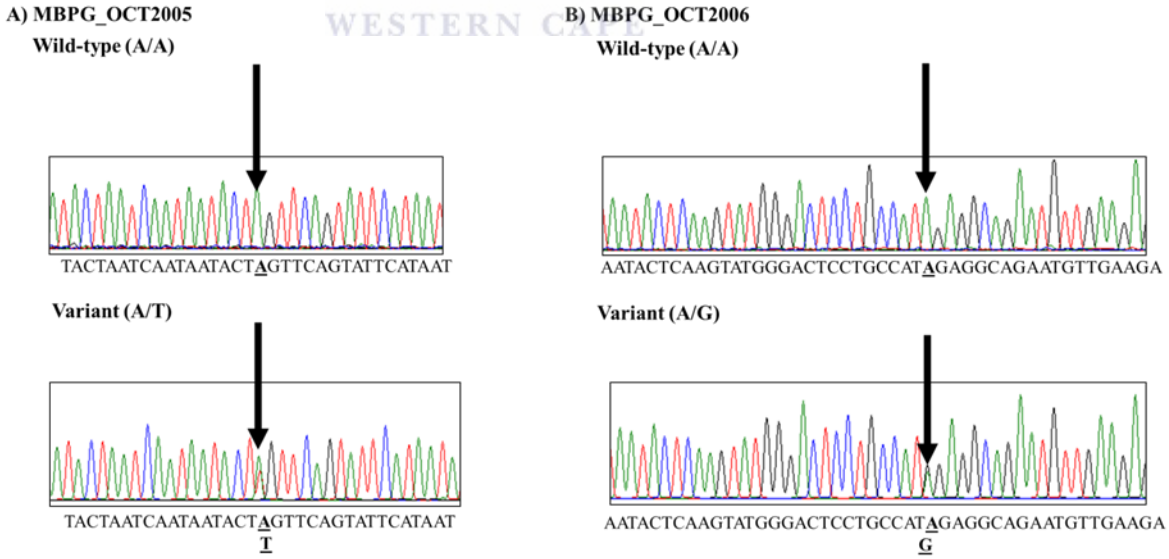

Figure 6.3 - Electropherograms of novel SNPs observed in the intronic region between exons 6 and 7 and exon 9 and 10 of *SLC22A2*. (A) MBPG_OCT2005 (wild-type MBPG_OCT2005A/A and variant MBPG_OCT2005A/T) a novel SNP detected in the intronic region between exons 6 and 7. (B) MBPG_OCT2006 (wild-type MBPG_OCT2006 A/A; variant MBPG_OCT2006G/C) a novel SNP detected in the intronic region between exons 9 and 10.

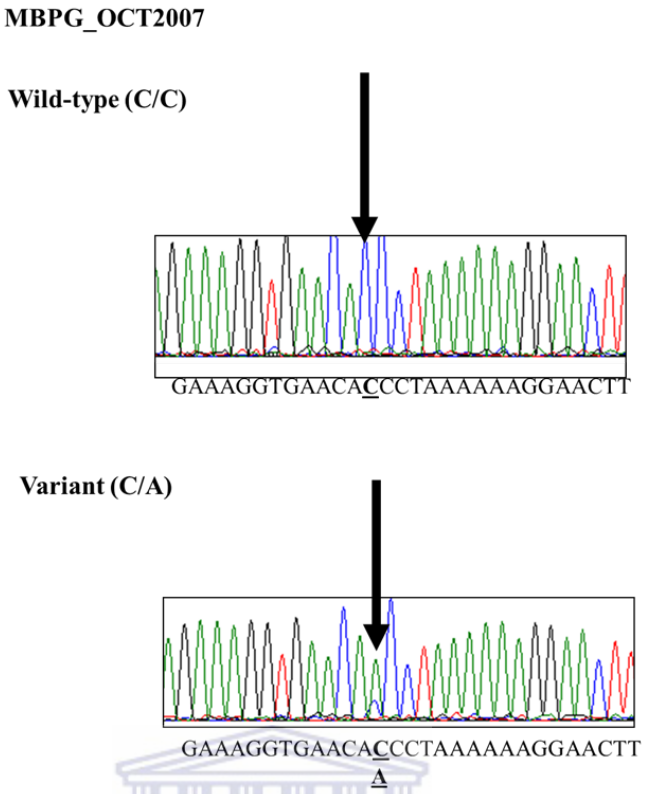

Figure 6.4 - Electropherogram of a novel SNP observed in the intronic region between exons 10 and 11of *SLC22A2*. MBPG_OCT2007 (wild-type MBPG_OCT2007C/C and variant MBPG_OCT2007C/A) a novel SNP detected in the intronic region between exons 10 and 11.

Fourteen of the 15 non-coding or intronic SNPs **(Table 6.2)** have been observed only in African populations or populations with an African connection. The MAF for nine SNPs is compared to other populations in **Table 6.3** and is depicted in **Figure 6.5** These variations were observed at MAFs between 0.5% and 26.3%. Moreover, seven novel SNPs (Figures 6.1 to 6.4), one in the 5′-untranslated region at position -156 from the start codon, with a MAF of 0.5%, and four in the intronic regions with MAFs between 0.1% and 0.5% were identified in this study. We also observed a singleton for rs112425400 a variant that has thus far only been observed in the Bushman of southern Africa. In addition, we also observed rs2279463, a MAF of 18.3%, a SNP that was identified as a risk locus in chronic kidney disease.

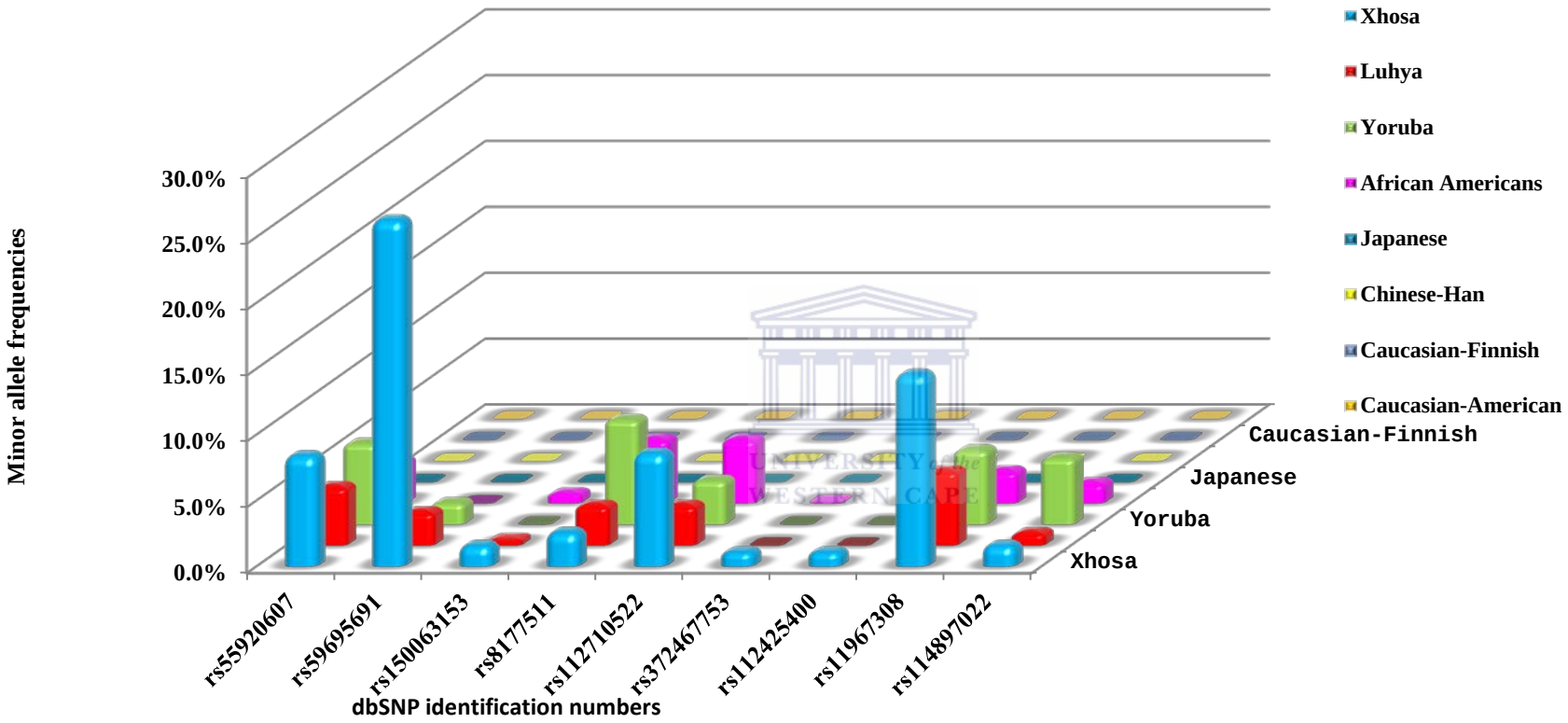

Figure 6.5 - Allele frequencies of selected *SLC22A2* SNPs in the Xhosa population compared to other African and world populations.

Table 6.3 - Comparison of MAF of selected *SLC22A2* SNPs identified by direct sequencing in the Xhosa population to other ethnic groups.

dbSNP ID	Minor Allele	Minor Allele Frequency (%)							
		Xhosa[a]	Luhya[b]	Yoruba[b]	African-American[b]	Japanese[b]	Chinese-Han[b]	Caucasian-Finish[b]	Caucasian-American[b]
rs55920607		8.4	4.5	6.2	3.3	0.0	0.0	0.0	0.0
rs59695691		26.3	2.6	1.7	0.0	0.0	0.0	0.0	0.0
rs150063153		1.6	0.5	0.0	0.8	0.0	0.0	0.0	0.0
rs8177511		2.6	3.1	8.0	4.9	0.0	0.0	0.0	0.0
rs112710522		8.6	3.1	3.4	4.9	0.0	0.0	0.0	0.0
rs372467753		1.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0
rs112425400		1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
rs11967308		14.6	5.7	5.7	2.5	0.0	0.0	0.0	0.0
rs114897022		1.6	1.0	5.1	1.6	0.0	0.0	0.0	0.0

a.This study; b. Data from 1000 Genomes

6.4.2. Haplotype Analysis

Haplotypes were calculated using an inferential procedure (Yong and Lin, 2005). This method identified 47 distinct haplotypes. The ten major haplotypes are listed in **Table 6.4.**the seven major haplotypes were observed at frequencies of 28.46%, 11.24, 7.00%, and 6.00% respectively.

Table 6.4 - Major haplotypes inferred from 28 *SLC22A2* SNPs identified with direct sequencing.

Haplotype ID	Haplotype	Frequency (%)
Haplotype *1	GCCTGGGTTTGCTGGGGCTACAAAGCCT	28.46%
Haplotype *2	GCCTGGGTTTGCTGCGGCTACAAAGCCT	11.24%
Haplotype *3	GCCTGGGTTTGCTGGGGCTACAAAGCTT	7.00%
Haplotype *4	GTCTGGGTTTGCTGGGGCTACAAAACCT	6.00%
Haplotype *5	GCCTGGGTTTGCCGGGGCTACAAAGCCT	2.26%
Haplotype *6	GCCTGGGTTTGCTGGGGCTACATAGCCT	2.17%
Haplotype *7	GCCTGTGCTTGCTGGGGCTACAAAGCCT	2.00%

6.5. Discussion

The kidney-specific hOCT2 plays an important role in the uptake of a wide range of OCs from the circulation and has been identified as a site of DDIs. The aim of this study was to determine the extent of the genetic variation in the *SLC22A2* gene, which codes for hOCT2, within the Xhosa population of South Africa. We have identified 28 SNPs in the *SLC22A2* gene, including seven novel variants, of 96 healthy Xhosa individuals.

Twenty-one of the SNPs identified in this study were already reported and listed in the dbSNP database. In a study by Leabman *et al.* (2002) 28 SNPs were also identified in ethnically diverse populations which included the African-American population (Leabman *et al.*, 2002). Among these five coding SNPs, T130T (rs624249), A270S (rs316019), R400C (rs8177516), K432Q (rs8177517), and V502V (rs316003), were also observed in this study. Leabman *et al*. (2002) went on to demonstrate that A270S (rs316019), R400C (rs8177516), and K432Q (rs8177517) are reduced-function variants that alter hOCT2 affinity for prototypical organic cations *in vitro*. In addition, they observed that the less frequent variants, R400C (rs8177516) and K432Q (rs8177517), resulted in significantly more and deleterious functional changes compared to the more frequently occurring A270S (rs316019). In another study Fukushima *et al*. (2004) identified 33 SNPs, including fourteen novel ones, in a group of 118 arrhythmic Japanese patients (Fukushima-Uesaka *et al.*, 2004). Only six of the variants observed were common among the Leabman and Fukushima studies. In this study we have observed nine SNPs (Figure 6.5), including seven novel variants, which are specific to African populations or populations with a link to the African continent and that were not observed by either the abovementioned studies.

Furthermore, we have identified a novel promoter SNP at position -156, which can potentially alter transcription of the *SLC22A2* gene. In addition, we also observed two promoter SNPs (rs59695691 and rs150063153) that are found only in African population groups. 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). However, on closer inspection of their results this essential region could probably be expanded to between -214 and -58 bp. 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 (USF-1) which functions as a basal transcriptional regulator of the gene. The effect of these African-specific variations on basal promoter regulation has not yet been determined and requires further investigation.

In addition to the novel promoter variant, we have also identified five intronic genetic variants novel. Although the functional impact of these intronic SNPs are currently unknown, the importance of non-coding variants in complex traits and human disease are increasingly being recognized (Ward and Kellis, 2012). Moreover, rare and low-frequency (MAF between 0.5 and 1.0%) variants, such as the novel polymorphisms identified in this study, have been hypothesized to explain a substantial fraction of the heritability of common, complex diseases (McClellan and King, 2010, Tennessen *et al.*, 2012).

Although this study revealed seven novel SNPs, the coding region of the *SLC22A2* gene of individuals investigated lacked the genetic diversity we expected to see in this population. Leabman *et al.* (2002) have ascribed the low mutational rate and amino acid substitutions observed with hOCT2 to selection pressure that has acted against amino acid changes within the transporter (Leabman *et al.*, 2002). According to them such selection may be due to the important function hOCT2 plays in the renal elimination of endogenous compounds and xenobiotics. Moreover, they have

concluded that amino acid residues in the TMHs tend to be evolutionary more conserved than those found in the loops of membrane transporters (Leabman *et al.*, 2003). This lack of amino acid and genetic diversity observed with hOCT2 and *SLC22A2* is not unique, and was also observed with hOCT3 and *SLC22A3* (Lazar *et al.*, 2003, Lazar *et al.*, 2008).

6.6. Conclusions

The kidney-specific hOCT2 plays an important role in the renal uptake of many commonly used clinical drugs. Moreover, hOCT2 is an important site of drug-drug interactions and is therefore clinically relevant. Several SNPs have been identified in the *SLC22A2* gene that alters the function of hOCT2 and consequently the pharmacokinetics/pharmacodynamics of its substrate drugs. This study represents the first report which investigated the presence of novel *SLC22A2* SNPs in the Xhosa population. The rare singleton SNPs identified and haplotypes inferred for the Xhosa population in this study is an important step in filling the gap with regards to genetic information on the pharmacogenetically relevant *SLC22A2* gene in indigenous southern African populations. The information generated in this study can potentially lay the foundation for future pharmacogenetic study design and for the identification of association between the rare SNPs and drug response and toxicity.

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CHAPTER 7

Summary and Future Perspectives

Membrane transporters of the SLC family, and specifically the OCTs and MATEs, 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. Moreover, the current body of knowledge suggest that these transporters also play an important part *in vivo* drug disposition, therapeutic efficacy, and adverse drug reactions. 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%. Genetic polymorphisms in *SLC22A1-3* and *SLC47A1* have been associated with reduced transport and efficacy of clinically important drugs such as, for example, the biguanide anti-diabetic metformin. 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, M440I and G465R) and Asians (P283L and R287G) were not only absent in the Xhosa subjects genotyped in this study, but are also absent from other African populations that are included in the HapMap and 1000 Genomes projects. Given that these variants have been implicated in reduced transport of the anti-diabetic metformin, screening for them in the Xhosa 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 (13.5%) and Africans (7.1%) compared to that observed for Caucasians (1.0%). On the other hand, the average reported MAF for A270S is higher for Africans (15.7%) than that recorded for Asians (13.3%), European-Caucasians (10.6%) 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 and V519F (*SLC22A1*) and R400C, K432Q, and I552N (*SLC22A2*) have to date not been reported for Asian or Caucasian populations or are very rare in these populations with MAF of <1%. Moreover, the *in vitro* and *in vivo* consequences of the V519F and I552N variants on hOCT1 and hOCT2 function,

respectively, 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 anti-diabetic 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 indigenous African populations still remain a priority.

Genotyping of the *SLC22A3* and *SLC47A1* genes, which encode hOCT3 and hMATE1 respectively, revealed that none of the nonsynonymous SNPs assayed were present in the Xhosa 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* and *SLC47A1* loci and pointed at the important physiological roles the hOCT3 and hMATE1transporters 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* and *SLC47A1* genes of Xhosa individuals.

In the current study we have identified seven novel SNPs in the *SLC22A2* gene of the Xhosa population. These novel SNPs include a variant in the 5′-untrnaslated region of *SLC22A2* -156 bp from the initiation codon. In addition, we have also observed two other promoter SNPs that have to date only been observed in African populations. We have searched the literature and to our knowledge no studies have

thus far investigated the impact of these substitutions on *SLC22A2* expression. The effect of the aforementioned promoter SNPs on the expression of *SLC22A2* and the turnover of hOCT2 needs to be investigated given the importance of hOCT2 in drug disposition and efficacy, as well as a site of DDIs. In addition, the correlation between genotype of the newly identified *SLC22A2* intronic SNPs and clinical phenotype also needs to be investigated.

The sample size of this study compared well to that of the HapMap 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 Xhosa population. However, sequencing whole genomes/exomes remains an expensive exercise.

To our knowledge this study is the first of its kind to investigate the allele frequencies of known SNPs in four pharmacogenetically relevant genes, *SLC22A1*, *SLC22A2*, *SLC22A3* and *SLC47A1*, in the Xhosa population. Furthermore, through sequencing of the promoter, exonic, and intronic flanking regions of *SLC22A2*, this study has revealed seven novel variants. This study again highlights the fact that not all African populations share the same allele frequencies of key pharmacogenes and, that care should be exercised in using a single African population as a proxy for all African populations in pharmacogenetic studies. In addition, a total of eight robust and cost-effective SNaPshot™ multiplex genotyping systems were developed and optimized for 78 *SLC22A1-3* and *SLC47A1* SNPs. 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 *SLC22A2* and *SLC47A1* 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.

Future studies require a more comprehensive sequencing of the *SLC22A1-3* and *SLC47A1-2* genes and to expand this to more individuals or, where such sequencing information becomes available from other large-scale 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 assessed in future work. In addition, the effects of the African-specific nonsynonymous V519F and I552N variants on transport kinetics, protein turnover, and plasma membrane localization of hOCT1 and hOCT2, respectively, also requires further investigation. Assessing of the genetic variation of SLC transporter genes with emerging clinical importance in the absorption and disposition of drugs such as *SLCO1B1*, *SLCO1B3*, *SLCO1A2*, *SLCO2B1*, *SLC15A1*, *SLC15A2*, *SLC22A6*, *SLC22A8* in the Xhosa and other indigenous African populations should also be prioritized in future studies.

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