GENETIC POLYMORPHISMS AFFECTING TACROLIMUS DOSE REQUIREMENTS IN GHANAIAN PATIENTS WITH END-STAGE RENAL DISEASE

BY

MICHAEL KOBINA OTABIL

(10283964)

THIS THESIS IS SUBMITTED TO UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF A MASTER OF PHILOSOPHY DEGREE IN PHARMACOLOGY

JULY, 2019

DECLARATION

I, Michael Kobina Otabil, do hereby declare that, the experimental work described in this research work was carried out by me under the supervision of Dr. William Kudzi of Centre for Tropical Clinical Pharmacology and Therapeutics, school of Medicine and Dentistry and Prof. Isaac Asiedu-Gyekye of Department of Pharmacology and Toxicology, School of Pharmacy, University of Ghana.

Michael Kobina Otabil Date: 28th April, 2020

Student (10283964)

Dr. William Kudzi Date: 28th April 2020

Supervisor

Prof. Isaac Asiedu-Gyekye Date: 28th April 2020

Supervisor

DEDICATION

I dedicate this research to God, my family and supervisors for the immense support and encouragement throughout my research.

ACKNOWLEDGEMENT

I wish to express my thanks to Dr. William Kudzi of Centre for Tropical Clinical Pharmacology and Therapeutics, School of Medicine and Dentistry, University of Ghana, for his scientific support, guidance and supervision. I thank him for providing me with an enabling working environment to carry out my research work.

My sincere thanks to Prof. Isaac Asiedu- Gyekye of Department of Pharmacology and Toxicology, School of Pharmacy, University of Ghana, for his continuous encouragement, valuable kindness and supervision.

To the staff of Centre for Tropical Clinical Pharmacology and Therapeutics, School of Medicine and Dentistry, Korle-Bu campus, I say thank you for welcoming me all the time in the research center.

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ABSTRACT

Background: ESRD is the irreversible damage of a person's kidney, affecting it normal physiological functions and rendering it fatal if not managed by dialysis or transplantation. In Ghana, ESRD is managed primarily by hemodialysis until the patient can afford kidney transplantation. However, transplants are accompanied with an immune response by the host immune system resulting in a high probability of organ rejection. Immunosuppressant dosing in combination with other immune regulatory medications are required to prevent transplant rejection. Tacrolimus is a first-line immunosuppressant; however, it has a narrow therapeutic range. Tacrolimus is a known substrate of CYP3A5, CYP3A4 enzymes and an efflux pump, P-glycoprotein. The differences in expression level of the substrates are a contributory factor to the individual variations of the pharmacokinetics of Tacrolimus.

General Aim: To determine the genetic polymorphisms in CYP3A5, CYP3A4 and MDR1 genes in Ghanaian patients with End Stage Renal Disease**.**

Method: This is a cross-sectional study that involved 87 ESRD patients, out of which 5 were transplant recipients and 82 were on dialysis. Clinical and demographic data were recorded and the genomic DNA isolated. Samples were genotyped for specific SNPs in *CYP3A5*3 6986 A>G* rs776746, *CYP3A4*1B -290 A>G* rs2740574, *MDR1_Ex12 1236 C>T* rs1128503, *MDR1_Ex26 3435 C>T* rs1045642, *MDR1_Ex21 2677 G>A* rs2032582 and *MDR1_Ex21 2677 G>T* rs2032582 using PCR-RFLP. The genetic frequencies of the transplant recipients were analyzed against the patients' Tacrolimus dose and trough levels.

Outcome: The mean age of study participants was recorded as 46 years ±14.39. The etiologies of ESRD were mostly hypertension and diabetes. The frequency of *CYP3A5*3 (6986 A>G)*

genes expressed showed that 4.6% were mutant. This mutant gene was expressed only among the dialysis patients. The frequency of *CYP3A4*1B (-290 A>G)* genes expressed showed that 79.31% were mutant, out of which 80% were from the transplant recipients. A wild-type genotypic frequency of 100% was recorded in the participants for the *MDR1_Ex12 (1236 C>T)* gene. Also, a 100% wild-type genotypic frequency was recorded in the participants for the *MDR1_Ex21 (2677 G>A)* genes. No homozygous mutant genotype was expressed for *MDR1_Ex21 (2677 G>T)* genes, however, 1.15% expressed the heterozygous genotype. None of the participants expressed the mutant genotype TT which affects the level of expressed P-glycoprotein in the transmembrane region of cells. The frequency of *MDR1_Ex26 (3435 C>T)* genes expressed showed that 1.15% were mutant genotype. From the Tacrolimus trough level and genotype analysis, four transplant recipients expressing the homozygous variant *CYP3A4*1B/*1B* recorded significantly lower Tacrolimus trough level (average of 5.95± 1.8 ng/ml) compared to the fifth recipient (10.3ng/ml). There was no clear correlation between the expressed genes of the MDR1 haplotype genes and Tacrolimus trough level.

LIST OF ABBREVIATIONS

ESRD	End-Stage Renal Disease
CKD	Chronic Kidney Disease
TAC	Tacrolimus
SNP	Single Nucleotide Polymorphism
MDR1	Multiple Drug Resistance Protein
ABCB1	ATP Binding Cassette Subfamily B-
CYP	Cytochrome P450
Ex	Exon
GFR	Glomerular Filtration Rate
HIV	Human Immunodeficiency Virus
KBTH	Korle Bu Teaching Hospital
ACEI	Angiotensin Converting Enzyme Inhibitor
ARB	Angiotensin Receptor Blocker
GHS	Ghana cedis
FKBP	FK506 Binding Protein
P-gp	P-glycoprotein
DNA	Deoxyribose Nucleic Acid

dsDNA	Double Strand Deoxyribose Nucleic Acid
RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris Acid Ethylenediaminetetraacetic acid
UPF	Undigested Polymerase Chain Restriction Fragment
WT	Wild-type
HT	Heterozygous
MT	Mutant
UV	Ultra-Violent
bp	Base Pair
FP	Forward Primer
RP	Reverse Primer

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

End-Stage Renal Disease (ESRD) is the irreversible damage of a person's kidney, affecting its normal function and rendering it fatal if not managed by dialysis or transplantation (Abbasi, 2010). ESRD is classified as stage 5 of the National Kidney Foundation Disease Outcomes Quality classification. Individuals diagnosed of ESRD have a severely reduced glomerular filtration rate with an estimated 10% of kidney cells functioning properly. This dysfunction causes fluid retention, dyslipidemia, anemia, disturbance of bone and mineral metabolism (Abbasi, 2010).

Global Burden of Disease statistical study in 2010, showed that 10% of the world's population are suffering from Chronic Kidney Disease and millions die each year (Dienemann et al., 2016). A study by Dienemann et al in 2016, documented that the annual mortality rate for every 100,000 people suffering from CKD in Ghana has increased by 10.6% since 1990, an average of 0.5% per year (Dienemann et al., 2016). In one study, it was found that the burden of ESRD is gradually increasing in Ghana, sub-Saharan Africa and Africa as a whole (Egbi et al., 2014). In Ghana, Hemodialysis and kidney transplantation are the kidney replacement therapy options available in treating ESRD condition.

Transplantation is necessitated to save the lives of patients suffering from kidney failure. However, transplants are accompanied with an immune response by the immune system and high probability of organ rejection. Immunosuppressant dosing in combination with other immune regulatory medication like glucocorticoid, mycophenolate mofetil is required to prevent

transplant rejection (Hesselink, 2005). Tacrolimus is a potent first-line immunosuppressant and plays a key role in preventing graft rejection in kidney transplant recipients. Tacrolimus is classified as a Calcineurin inhibitor drug. The narrow therapeutic range and individual variation in dose requirements of Tacrolimus are important issues in clinical setting (Hesselink, 2005).

Tacrolimus is a known substrate of the liver cytochrome P450 (CYP) 3A5 and 3A4 enzymes and a transmembrane protein efflux pump, P-glycoprotein. The difference in expression level of these enzymes and the protein pump as a result of single nucleotide polymorphism (SNPs) in the genes coding them are a contributory factor to the individual variations of the pharmacokinetics of Tacrolimus. Tacrolimus use is associated with serious side effects, primarily nephrotoxicity (Hesselink, 2005). Presently, it is not clear whether the parent drug, Tacrolimus metabolites or both are the cause of the nephrotoxicity side effect of Tacrolimus dosing. Other adverse side effects include hypertension, insomnia, nausea, chronic heart failure, constitutional aplastic anemia, malignant lymphoma, Stevens-Johnson syndrome in some patients (Niioka et al., 2013).

1.2 Problem statement

Kidney malfunction is a worldwide problem affecting about 10% of world's population, with millions of recorded death due to inefficient treatment (Dienemann et al., 2016). According to 2010 Global Burden of Disease Study, death from kidney malfunction was ranked 27th in 1990 and 18th in 2010 (Dienemann et al., 2016).

A worrying statistic from the National Kidney Foundation Ghana over the past few years shows a continual increase in reported kidney cases in Ghana. Reported kidney malfunction cases from Korle-Bu Teaching Hospital shows 2,435 cases in 2008, 2,387 cases in 2009, 2,593 cases in 2010, 2,687 cases in 2011 and 3,612 cases in 2012 (Ephraim et al., 2015). Annual report of

Korle-Bu Teaching Hospital (2016) showed that renal failure was the second largest cause of death, accounting for 13.95% recorded mortalities (KBTH, 2016).

Diabetes and hypertension are the commonest causes of CKD in Ghana. The rising incidences of these diseases have simultaneously increased the incidence of CKD and as such ESRD. Kidney transplantation is gradually being integrated into the Ghanaian healthcare system, and in the not too distant future it is likely to be the mainstay of treatment for ESRD in Ghana. Kidney transplant recipients require adequate immunosuppression in order to prevent graft rejection.

Tacrolimus is a key component of the immunosuppression protocol used to manage post-transplant graft rejection. Tacrolimus is a known substrate of cytochrome CYP (P450) 3A5 and 3A4 enzymes and a transmembrane protein efflux pump, P-glycoprotein. The differences in expression level of these enzymes and P-glycoprotein due to SNP in the genes coding them are a contributory factor to the individual variations of the pharmacokinetics of Tacrolimus. Unfortunately these genes are not genotyped before Tacrolimus is administered to transplant patients, and the initial dose administered every 12 hours are changed based on how patients response to it.

The effect of SNPs in these genes and their association to Tacrolimus dose in Ghanaian populace has not been reported in literature. There is no pharmacogenomics data or information on Tacrolimus metabolism in the Ghanaian population to assist clinicians to properly manage kidney transplant recipients in Ghana. Therefore the result from this study will be of clinical relevance to successful management of kidney transplant recipients in Ghana (Bruckmueller et al., 2015).

1.3 Justification

Current immunosuppressive therapy after kidney transplant is aimed at suppressing the host immune reaction using a combination of several drugs that functions on multiple pathways of the immune response. A first line effective immunosuppressant is Tacrolimus but its use is associated with adverse effects in different individuals (Bruckmueller et al., 2015).

Study by Robert et al in 2005, indicates that variation in Tacrolimus blood concentration of 5-10ng/ml is associated with either a reduced therapeutic effectiveness or toxicity; genetic variations in CYP3A5 and CYP3A4 enzymes and a protein pump P-glycoprotein contributes to Tacrolimus blood concentration in kidney transplant recipients (Atkins, 2005).

The effect of SNPs in CYP3A4 and CYP3A5 enzymes and a protein efflux pump MDR1 on Tacrolimus dose has not been described, reported and documented in the Ghanaian population to help nephrologist or clinicians in managing Tacrolimus administration in kidney transplant recipients or ESRD patients who are been prepared for kidney transplant.

1.4 Aim

To determine the genetic polymorphisms in CYP3A5, CYP3A4 and MDR1 genes in Ghanaian patients with End Stage Renal Disease.

1.5 Specific objectives

1. To determine the genetic frequencies of *CYP3A4*1B*, *CYP3A5*3* and *MDR1 (Ex12-1236 C>T, Ex21-2677 G>T, Ex21-2677 G>A, Ex26-3435 C>T*) polymorphisms among End Stage Renal Disease patients.

Hypothesis: There are different genotypic frequencies of CYP3A4, CYP3A5 and MDR1 alleles expressed among kidney transplant recipients.

2. To investigate the association between SNPs of *CYP3A5*3* and the trough blood concentration of Tacrolimus in Ghanaian kidney transplant recipients.

   Hypothesis: Patients with *CYP3A5*3* SNPs will have higher

   Trough blood Tacrolimus levels per dose.

3. To investigate the association between SNPs of *CYP3A4*1B* and the trough blood concentration of Tacrolimus in Ghanaian kidney transplant recipients`.

   Hypothesis: Patients with *CYP3A4*1B* SNPs will have lower trough blood Tacrolimus levels per dose.

4. To investigate the association between SNPs of MDR1 and the trough blood concentration of Tacrolimus Ghanaian kidney transplant recipients.

   Hypothesis: Patients with MDR1 SNPs will have higher trough blood Tacrolimus levels per dose.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 End-Stage Renal Disease

The kidney plays a central role in maintaining the body's homeostasis, and it does this by regulating body fluid, electrolyte and acid-base levels (Kim et al., 2012). CKD is an abnormality in the kidney structure and or function, present for more than 3 months (Stevens et al., 2014). Progressive kidney disease gradually leads to the common end point characterized by shrinking and fibrosis; with only 10% of the kidney tissue properly functioning well. This is called End-Stage Renal Disease. There are five stages in CKD based on the efficiency of its glomerular filtration rate. Stage 5 of CKD is referred to as ESRD and that is the stage when only about 10% of the kidney cells are functioning properly. The stages of CKD are shown in table 2.1

Table 2. 1: The 5 stages of Chronic Kidney Disease

Stage	Description	eGFR (mL/min)	Potential complications of reduced GFR (in alphabetical order)
1	Kidney damage with normal or ↑ GFR	≥90	• Anemia, including functional iron deficiency • Blood pressure increases • Calcium absorption decreases • Dyslipidemia /heart failure/volume overload • Hyperkalemia • Hyperparathyroidism • Hyperphosphatemia • Left ventricular hypertrophy • Metabolic acidosis • Malnutrition potential (late)
2	Kidney damage with mild ↓ GFR	60–89	
3	Moderate ↓ GFR	30–59	
4	Severe ↓ GFR	15–29	
5	Kidney failure	<15 or dialysis	

Source: Adapted from Identification, Evaluation and Management of Chronic Kidney Disease T (www.health.gov.bc.ca/gpac/pdf/ckd.pdf)

2.1.1 Prevalence of End-Stage Renal Disease

In Ghana, it's estimated that CKD affect about 10.4% in Ghanaian population CKD has been recorded to account for 5% and 9% medical admissions in Ghana and Nigeria respectively (Ephraim et al., 2015). Ephraim et al in 2015, found CKD prevalence of 46.8% among hypertensive in an outpatient setting in Accra, Ghana (Afolabi & Abioye, 2009).

Studies showed that CKD is one of the most common complications in hypertensive and diabetes mellitus (Judd & Calhoun, 2015; Plange-Rhule et al., 1999). In another study they recorded a prevalence of 44% in hypertensive patients, 16% on obese patients, 39% in diabetic patients and 12% in patients diagnosed with Human Immunodeficiency Virus (HIV) (Ephraim et al., 2015). A study by Atkins et al showed that among patients diagnosed with CKD, approximately 30% of them progress to ESRD (Atkins, 2005). In Ghana, 2016 annual report from Korle Bu Teaching Hospital showed that the prevalence rate of ESRD is 1.6% (KBTH, 2016).

2.2 Treatment of Chronic Kidney Disease

CKD is categorized into 5 different stages as stated in the Kidney Disease Quality Outcome Initiative (KDOQI) classification chart (Stevens et al., 2014). Knowledge of both the disease stage and contributing factors of CKD in each patient is key to patient's care. Stages 1 to 4 of CKD are usually managed conservatively to slow down the rate of disease progression. This is done by adequately managing causative factors like diabetes, hypertension. When glomerular filtration rate falls below 10ml/min/1.73m^2, uremic toxins (serum urea and creatinine), hydrogen and potassium ions accumulate heavily in the kidney tissue, destroying about 90% renal cells. At this stage the CKD is said to have developed into ESRD and can cause death if not managed by dialysis, preferably 3 times a week until successful transplantation.

Treatment of CKD is directed at managing disease conditions such as hypertension. Angiotensin Converting Enzyme Inhibitor drugs (ACEI) or Angiotension Receptor Blocker drugs (ARB) such as spironolactone, benazepril, fosinopril, quinapril, are the first line antihypertensive therapy agents. Angiotensin which is produced to restore blood flow rate tends to cause greater vasoconstriction in efferent arteriole than afferent arteriole, resulting in increased tension on the glomerular. With time the structural nature and functional role of glomerular is destroyed. Also management of comorbid conditions like diabetes, hyperlipidemia, and cardiovascular disease among patients with CKD is helpful in treating CKD (Afolabi & Abioye, 2009). An effective control of blood sugar level with metformin, glipizide, in both type 1 and type 2 diabetes reduces the risk of developing diabetic nephropathy. Statin is another effective drug used in managing CKD, as it is used effectively in cardiovascular disease. Cardiovascular disease is recorded as one of the leading cause of death among CKD patients as a recent study showed that early stage of CKD is characterized with cardiovascular events and death (Afolabi & Abioye, 2009). Patients showing deteriorating kidney state or renal insufficiency are referred to the renal clinic for management and if no progress is made, dialysis becomes the primary option to save their lives (Thomas et al., 2008).

Dialysis machine is usually used to remove uremic toxins from the blood. Dialysis is available mainly as peritoneal dialysis and hemodialysis. In Ghana, the commonest type found is hemodialysis. Ghanaian patients diagnosed with ESRD have the option of hemodialysis and renal transport. Hemodialysis in Ghana is very expensive and a section cost about GHS800.00; a patient is expected to attend at least 2 sections per week. A study done in Nigeria by Okunola et al in 2013 showed that out of 180 patients diagnosed of ESRD, only 100 are able to afford dialysis therapy for more than 3 months (Okunola et al., 2013). The cost of dialysis is not

covered by the Ghana National Health Insurance Scheme and as such with the exception of few people who are able to afford dialysis, majority find it expensive and as such either stay away from receiving treatment or resort to self-medication.

Treatment of ESRD is variable among CKD patients in Africa and very limited literature on availability of dialysis are available in West Africa (Naicker, 2003). The overwhelming cost of dialysis and kidney transplant coupled to the 10 years average life expectancy of transplant recipient calls for the need to develop new chronic dialysis programs that have short dialysis section per week; also develop post-transplant regimen that are cheap, efficient and can increase the lifespan of a kidney transplant recipient.

2.3 Immunosuppression of kidney transplant patients

Graft rejection is the main complication that affects the success of kidney transplantation. This can occur immediately after transplant (Hyperactive rejection) or any time from the first week after transplant (Acute rejection) or take place over many years (Chronic rejection). The host's immune system is the key player in graft rejection, and as such the best way in managing and preventing graft rejection is the administration of immune-regulators like immunosuppressive that regulate the host's immune system. Immunosuppressants given to patients include Basiliximab, Methylprednisone, Mycophenolate mofetil, Tacrolimus and Cyclosporine.

Immunosuppressive therapy is aimed at reducing the possibility of graft rejection and regulating adverse effects resulting from over suppression of host immune system, such as malignancy, infections and cardiovascular risk factors. Immunosuppressive therapy combines immunosuppressant that have different mechanisms of action or target points, to obtain a synergistic effect with optimum immune regulation to minimize any undesirable effects.

Immunosuppressant therapy protocol used in managing post-kidney transplant immune response varies widely among centers but the basic principle behind immune suppression is common across. Immunosuppression therapy occurs in two phases; namely Induction and Maintenance.

2.3.1 Induction phase:

Induction phase is the initial immunosuppression therapy that takes place during the first few days or weeks after kidney transplantation. It's usually intense and drugs used are either monoclonal or polyclonal antibodies (e.g. Basiliximab) and are either lymphocyte depleting or non-lymphocyte depleting. This therapy has been used over the years by nephrologists with varying degree of success recorded in different renal centers (Okunola et al., 2013). Within 24h of allograft, initiation of therapeutic agents begins. An initial Tacrolimus dose of 0.2mg/kg is given every 12h. A trough concentration from 10ng/ml to 15ng/ml is targeted in the period of 0 to 3 months after transplant. Nephrologist at the Renal Unit in Korle Bu Teaching Hospital initiate with intravenous Methylprednisone 1g and intravenous Basiliximab 20mg on day 0 and 20mg on day 4.

2.3.2 Maintenance phase:

This is the immunosuppressive therapy given following induction phase. It's aimed at suppressing the immune system to prevent graft rejection to prolong the lifespan of kidney transplant recipients. Tacrolimus and Cyclosporine are the main immunosuppressant used in this phase. After 3 months of transplant and Tacrolimus dosing, a trough concentration of 5ng/ml to 10ng/ml is maintained for maximal concentration to avoid organ rejection and toxicity. In Ghana, nephrologist at the Renal Unit in Korle-Bu Teaching Hospital follow the protocol of

administering 0.075mg/kg oral Tacrolimus every 12 hours, 1g of oral Mycophenolate mofetil every 12 hours and 10mg oral Prednisone every 12 hours.

2.4 Mechanism of action of Tacrolimus

Tacrolimus is a potent first-line immunosuppressant that has a narrow therapeutic index. It has variable absorption rate with peak blood or plasma concentrations being reached in 0.5 to 6 hours; approximately 25% of the oral dose is bioavailable. It is extensively bound to red blood cells, with a mean blood to plasma ratio of about 15; albumin and alpha 1-acid glycoprotein appear to primarily bind tacrolimus in plasma. Tacrolimus is completely metabolized prior to elimination. The mean disposition half-life is 12 hours and the total body clearance based on blood concentration is approximately 0.06 L/h/kg. The elimination of tacrolimus is decreased in the presence of liver impairment and in the presence of several drugs. Tacrolimus binds to a family of proteins called FK506 binding proteins (FKBPs). This binding forms a large pentameric complex consisting of Tacrolimus, FKBP, calcineurin, Ca^{2+} and calmodulin. The formation of this complex causes the inhibition of the phosphatase activity of calmodulin proteins (McKeon, 1991). The inhibition of the calcineurin phosphatase activity inhibits the transport of transcription factors to the cell nucleus and as a result blocks T-cell activation, differentiation and proliferation. Tacrolimus is administered orally and intravenously after transplant to reduce the risk of organ rejection, increase grafting and recipient survival (Chen et al., 2013).

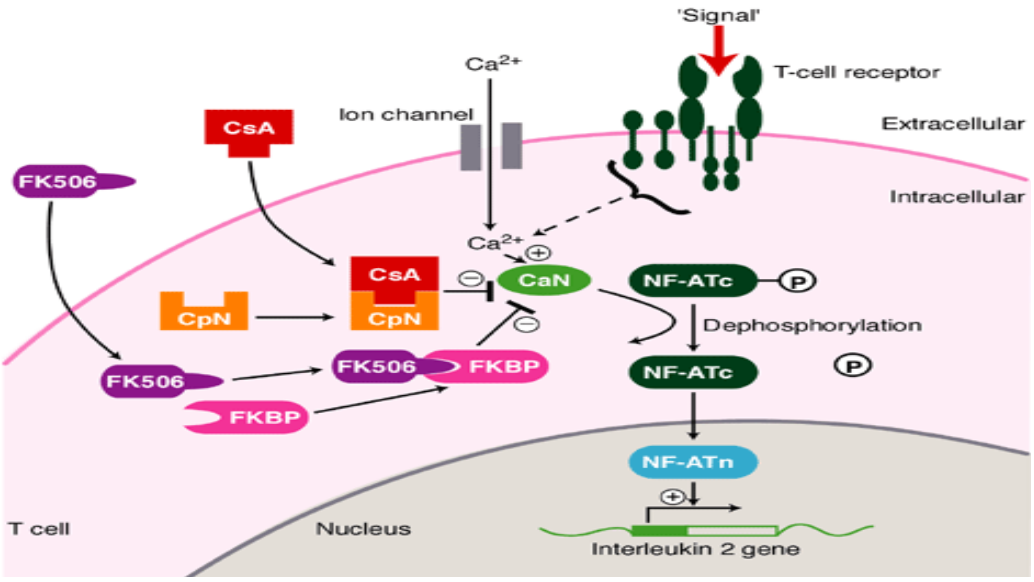

Figure 2. 1: Mechanism of action of Tacrolimus in the cell.

Source: Expert Reviews in Molecular Medicine@2000 Cambridge University Press

2.5 Genetic Polymorphisms affecting drug metabolism

2.5.1 Polymorphisms in C4YP3A4 and CYP3A5 enzymes

Pharmacokinetic studies have shown irregular and variable absorption of Tacrolimus from the gastrointestinal duct. The in inter-individual variations in its kinetics in kidney transplant recipients. Other pharmacokinetic studies indicate that Tacrolimus binds to plasma protein albumin and α-1-acid glycoprotein, as well as erythrocytes. Due to the high binding of Tacrolimus to plasma contents, whole blood is used to study or analyze Tacrolimus kinetics (Lo et al., 2011).

Extensive researches have been done to study the effect of inter-individual variations of metabolizing C4YP3A4 and CYP3A5 caused by SNP on Tacrolimus dose, SNP in interleukin 6 and 10 contributing to metabolizing the drug in recipient (Picard & Marquet, 2011). These and a

few more research findings had led researchers conclude that genetic polymorphisms could possibly be a key contributing factor to Tacrolimus metabolism and associated adverse effects (Elens et al., 2011).

Blood concentration of 5-10ng/ml Tacrolimus is considered the optimal concentration to avoid rejection. Too low trough concentration causes reduced therapeutic effectiveness whiles a higher trough concentration causes drug toxicity. The main determinant of Tacrolimus blood concentration is a SNP in intron 3 of CYP3A5 (6986 A>G) rs776746; also known as *CYP3A5*3*). The mean dose-adjusted blood Tacrolimus concentration was significantly higher among CYP3A5*3/*3 homozygotes than the carrier of the wild-type allele (Lo et al., 2011). Also, inter-individual variability in CYP3A4 activity affects Tacrolimus concentration. This is usually due to differences in transcript levels as a result of nucleotide changes in the promoter region (Guengerich, 2002). *CYP3A4*1B* gene is a common allele located in the promoter region, is associated with differences in transcriptional activity. This causes an increased hepatic expression of CYP3A4 enzymes.

2.5.2 Polymorphism in pump P-glycoprotein (P-gp) MDR1 gene

The MDR1 gene codes for the protein P-glycoprotein. P-glycoprotein functions as a protein pump that drives the efflux of many drugs in an ATP-dependent fashion. Expression of *MDR1* are found in adrenal gland, large and small intestinal epithelium, kidney, placenta, liver, peripheral blood lymphocytes and capillary endothelial cells of brain and testes (Lo et al., 2011). In earlier study, *MDR1* was identified to be responsible for the resistance of cancerous cells to many anticancer agents. MDR1 in trophoblast function by blocking the transfer of hydrophobic xenobiotics/drugs across the human placenta.

2.5.2.1 *MDR1* gene structure and site of polymorphism

The P-gp gene is located on the chromosome 7, having 28 exons that encode a protein of 1280 amino acids. *MDR1* is also called ATP-Binding Cassette (ABC) transporter superfamily because it has a highly conserved ATP-binding site in two homologous halves and the linker region (Ieiri et al., 2004). SNP identified includes a non-synonymous mutation in which there is a G→T and G→A substitution at position 2677 in exon 21 (G2677T and G2677A). This leads to a change in amino acid from Ala at codon 893 (Ala893) to Ser and Thr, respectively. A study carried out found that homozygous mutant TT genotype was associated with reduced expression of intestinal *MDR1* proteins when compared with homozygous wild-type CC genotype (Hoffmeyer et al., 2000). In another study, the absolute bioavailability from both oral and intravenous administrations of digoxin was significantly higher in expressers of variant allele T (Ieiri et al., 2004).

A C→T change at position 3435 in exon 26 (C3435T) does not change the amino acid sequence. Other polymorphisms like C1236T and C3396T are also silent and do not cause a change in protein sequence (Masuda et al., 2006). Despite the fact that a polymorphism at 3435 in exon 26 induces a 'silent' (synonymous) mutation, it's been found to alter P-gp functions (Wolking et al., 2015).

Polymorphism in P-gp directly affect the amount of drug reaching systemic circulation as well as the site of action (Liao et al., 2017). A study by Masuda et al in 2006 showed a high correlation between the role of P-gp expression and Tacrolimus bioavailability, whilst others reported conflicting results (Masuda et al., 2006).

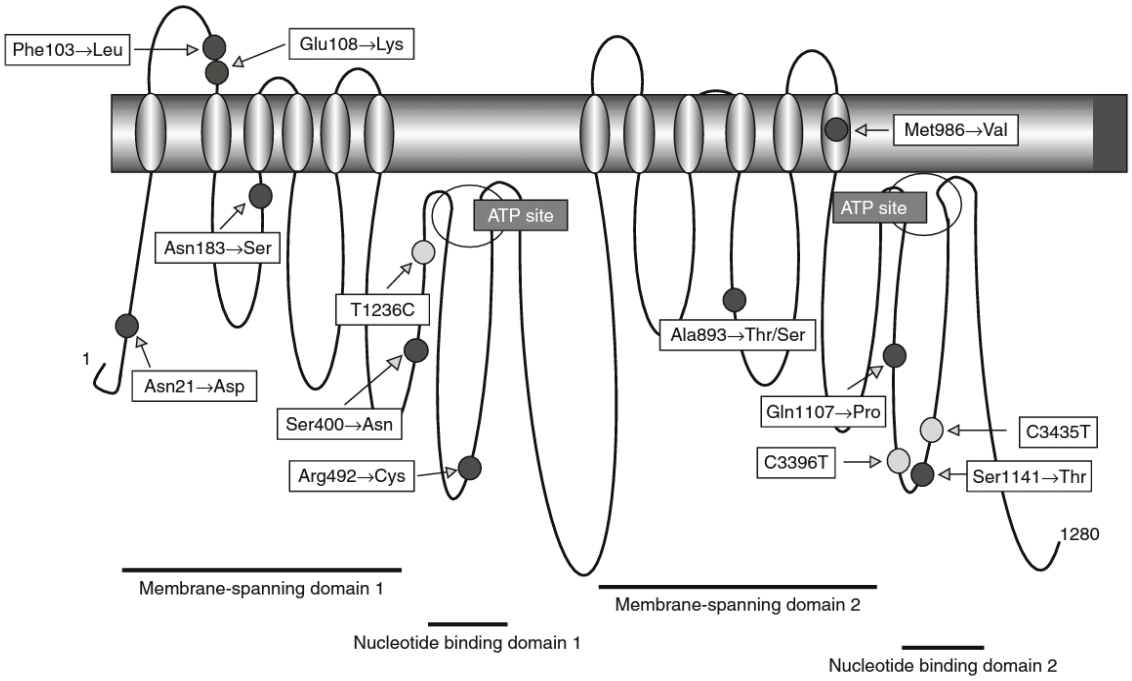

Figure 2. 2: Secondary structure of P-glycoprotein.

Source: Expert Reviews Molecular Medicine

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Reagents and Equipment

The equipment, reagents and preparations of solutions used in the study are described in Appendix I and Appendix II. Primer sequences are shown in Appendix III.

3.2 Study design

This was a cross-sectional study which involved Ghanaian patients with ESRD receiving dialysis and patients on oral Tacrolimus administration after successful kidney transplant.

3.3 Study sites

The study **was** conducted at the Renal Unit of Korle Bu Teaching Hospital (KBTH). KBTH is a referral hospital with over 1800 beds for in-patients and has several Specialist clinics, Wards, Pharmacies and Reference laboratories. The Renal Unit is a subspecialty unit under the department of medicine. Specialists review kidney transplant recipients during the Kidney transplant Clinic, which is held at the renal unit on Fridays, ESRD patients on hemodialysis are reviewed at the Dialysis clinic, which is also held at the renal unit on Wednesday mornings. The general Nephrology clinic is held at the Central OPD on Monday afternoons, this is where ESRD patients who are not on dialysis have their follow up care. Ethical clearance for the study was obtained from the Ethical and Protocol Review Committee of the University of Ghana, College of Health Sciences with reference number CHS-Et/M.1- P2.8/2016-2017.

3.4 Study population

The participants recruited into this study gave their informed consent (Appendix IV) after protocol approval by the Ethical and Protocol Review committee, College of Health Sciences, University of Ghana. The recruitment involved a non-probability convenient sampling where only patients who willingly accepted to participate in the study were recruited. The questionnaire administered is described in Appendix V.

3.5 Inclusion and exclusion criteria

3.5.1 Inclusion criteria

1. Patients who have had a single renal allograft.
2. End Stage Renal Disease patients receiving dialysis.
3. Transplant patients who are on immunosuppressive drug Tacrolimus for more than three months.

3.5.2 Exclusion criteria

1. Patients with multiple organ transplant.
2. Patients taking other medications that may interact with Tacrolimus.

3.6 Sample size determination

The minimum number of study participants for this cross sectional study was calculated as described Pourhoseingholi et al (Pourhoseingholi et al., 2013). Using the prevalence of 1.6% for End Stage Renal Disease in Ghana as documented by Korle Bu Teaching hospital's annual report

(KBTH, 2016), a confidence interval of 95% and a precision of 3.2%, a minimum of 59 study participants was calculated as the minimum sample size for the study.

However, a total of 87 participants were enrolled in the study. End Stage Renal Disease patients on dialysis were 82 and End Stage Renal Disease patients who have undergone kidney transplant were 5.

3.7 Pilot Study

Pretesting was done at the Cardiothoracic Unit (dialysis section) of the Korle Bu Teaching Hospital. A total of 10 dialysis patients were recruited into the study**.** None of the patients had received kidney transplant. The purpose of the research was explained to each patient and 10 patients agreed to be recruited into the study after 14 days of visiting the Unit. Patients were carefully guided through all the questions in the questionnaires and their responses were recorded. Blood was not sampled from the patients. Responses from the patients were analyzed and discussed with a Clinical Pharmacologist (Research supervisor) and two other nephrologists at the Renal Unit of the department of Medicine and Therapeutics, Korle Bu Teaching Hospital.

3.8 Genomic DNA extraction and Quantification

3.8.1 Genomic DNA extraction

Genomic DNA was extracted from whole blood of 87 samples using a commercial extraction kits; Zymo Research Quick-gDNA MiniPrep kits (Inqaba Biotec Ltd, SA) following the manufacturer's protocol. Frozen whole blood preserved in EDTA tubes were thawed on an electric blood roller at room temperature for about 30 minutes. Whole blood (100µL) was

pipetted into Eppendorf tubes and 400µL of Genomic lysis buffer was added. The mixture was vortex for 6 seconds and allowed to stand for 10 minutes at room temperature. The mixture was transferred to a Zymo- Spin Column in a collection tube and centrifuged at 10,000×g for 1 minute. The collection tube was discarded and the Zymo- Spin column transferred to a new collection tube. DNA Pre- Wash buffer (200µL) was added to the Spin column and centrifuged at 10,000×g for 1 minute. gDNA Wash buffer (500µL) was added to the Spin column and centrifuged at 10,000×g for 1 minute. The Spin column was transferred to a clean Eppendorf tube and 50µL DNA Elution buffer was added to the Spin column and allowed to stand on the working bench for 5 minutes at room temperature. Mixture was centrifuged at top speed (14,000×g) for 30 seconds to elute DNA into the Eppendorf tube. The eluted DNA was stored at -20^{O}C and used to genotype the specific alleles.

3.8.2 Spectrophotometric Quantification of DNA

From the Home screen of a Nanodrop Lite instrument (Thermo Scientific Inc.), double stranded DNA (dsDNA) assay was selected. Water (1µL) was pipetted onto the lower pedestal, upper arm lowered and Blank pressed on the screen. Arm was raised and both pedestals cleaned with dry laboratory wipe. DNA (1µL) sample was pipetted onto the lower pedestal, upper arm closed and "Measure" pressed from the screen. DNA quantification was done in batches and the average DNA concentration was used to calculate the volume of DNA to be used for PCR.

3.9 Genotyping

Genotyping of the specific alleles *CYP3A5*3 (6986 A>G), CYP3A4*1B (-290 A>G), MDR1_Ex12 (1236 C>T), MDR1_Ex26 (3435 C>T), MDR1_Ex21 (2677 G>A), MDR1_Ex21 (2677 G>T)* were performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) as previously described by Sarasamma et al. (2016), with some modification after optimizing the protocols (Sarasamma et al., 2016). The first step in a PCR-RFLP analysis was to amplify the DNA sequence of interest by PCR. For each PCR reaction, the DNA sequence of interest was amplified using specific primers designed to anneal to regions of the DNA sequence. Primer sequences used in amplifying the genes of interest are shown in Appendix III. This was followed by digestion of the PCR product (amplicon) with an appropriate restriction enzyme. After restriction digest of the DNA sequence, the digest was subjected to electrophoresis to separate the DNA into fragments of different sizes based on the presence or absence of a restriction enzyme recognition site on the DNA. Results from the electrophoresis were viewed on a Gel Imager.

3.9.1 Identification of *CYP3A5*3 (6986 A>G)* genotypes by PCR-RFLP

PCR for *CYP3A5*3 (6986 A>G)* gene was performed in a 20µL total reaction volume. The reaction mixture was made up of nuclease-free water, 10µM forward (F) primer, reverse (R) primer, One Taq Quick-Load 2X Commercial Master Mix Standard Buffer and genomic DNA. The details of this PCR mixture and PCR cycling conditions are contained in Tables 3.1 and Table 3.2 respectively. A negative control reaction was prepared that contained all other reagents except genomic DNA.

Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 5µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 75V for 45minutes and then visualized with a Gel Imager (Bio-Rad Laboratories, Inc). The expected PCR product size is 293bp.

Aliquots of the PCR products were digested with appropriate restriction enzyme (Ssp I) in a 20µL reaction mix containing nuclease-free water, Buffer G and Ssp I restriction enzyme as shown in Table 3.3. The reaction mixture was prepared in a sterile Eppendorf tube and incubated for 3 hours at 37^{O}C. Agarose gel (3% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 10µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 85V for 60minutes and then visualized with a Gel imager.

Expected fragments sizes represented in fig. 3.1 are 148bp, 125bp and 20bp for wild-type allele; 168bp, 148bp, 125bp and 20bp for heterozygous, 168bp and 125bp for mutant allele.

Table 3. 1: *CYP3A5*3 (6986 A>G)* PCR reaction mixture

Reagents	**Volume (µL)**
Nuclease-free water	1
Forward primer	2
Reverse primer	2
Master Mix	10
Genomic DNA	5
Total	20

Table 3. 2: *CYP3A5*3 (6986 A>G)* gene PCR cycling conditions

Conditions	**Temperature/^{O}C**	**Time**	**Number of Cycles**
Initial denaturation	94	7min	1
Denaturation	94	1min	35
Annealing	58	1min	
Extension	72	1min	
Final extension	72	7min	1

Table 3. 3: Restriction digest reaction mixture for *CYP3A5*3 (6986 A>G)* gene

Reagents	**Volume (µL)**
Nuclease-free water	7
10x buffer G	2
Ssp I	1
PCR product	10
Total	20

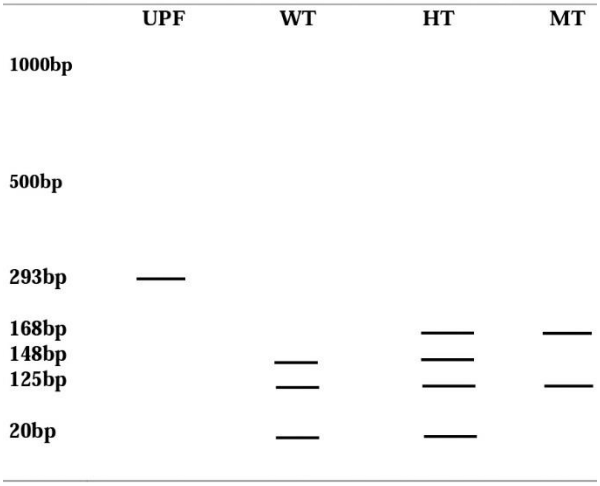

Figure 3. 1: A schematic diagram of PCR-RFLP electrophoregram of *CYP3A5*3 (6986 A>G)* allele.

Where UPF is undigested PCR product, WT is Wild-type (A), HT is heterozygous (A/G) and MT (G) is mutant.

3.9.2 Identification of *CYP3A4*1B (-290 A>G)* genotypes by PCR-RFLP

PCR for *CYP3A4*1B (-290A>G)* gene was done in a 20µL total reaction volume. The reaction mixture was made up of nuclease-free water, 10µM forward (F) primer, reverse (R) primer, One Taq Quick-Load 2X Commercial Master Mix Standard Buffer and genomic DNA. The details of this PCR mixture and PCR cycling conditions are contained in Tables 3.4 and Table 3.5 respectively. A negative control reaction was prepared that contained all other reagents (nuclease-free water, 10µM forward (F) primer, reverse (R) primer, except genomic DNA.

Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 5µL of the PCR products into each well in the agarose

gel. The setup was electrophoresed at 75V for 45minutes and then visualized with a Gel imager. The expected PCR product size is 334bp.

Aliquots of the PCR products were digested with appropriate restriction enzyme (Pst I) in a 20µL reaction mix containing nuclease-free water, Buffer O and Pst I restriction enzyme as shown in Table 3.6. The reaction was prepared in a sterile Eppendorf tube and incubated for 3 hours at 37^{O}C. Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 10µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 85V for 60minutes and then visualized with a gel imager.

Expected fragments sizes represented in fig. 3.2 are 220bp, 81bp and 33bp for wild-type allele; 220bp, 199bp, 81bp, 33bp and 21bp for heterozygous, 199bp, 81bp and 21bp for mutant allele.

Table 3. 4*: CYP3A4*1B (-290 A>G)* gene PCR reaction mixture

Reagents	**Volume (µL)**
Nuclease-free water	1
Forward primer	2
Reverse primer	2
Master Mix	10
Genomic DNA	5
Total	20

Table 3. 5: *CYP3A4*1B (-290 A>G)* gene PCR cycling conditions

Conditions	**Temperature/^{O}C**	**Time**	**Number of Cycles**
Initial denaturation	94	7min	1
Denaturation	94	1min	35
Annealing	58	1min	
Extension	72	1min	
Final extension	72	7min	1

Table 3. 6: Restriction digest reaction mixture for *CYP3A4*1B (-290 A>G)* gene

Reagents	**Volume (µL)**
Nuclease-free water	7
10X buffer O	2
Pst I	1
PCR product	10
Total	20

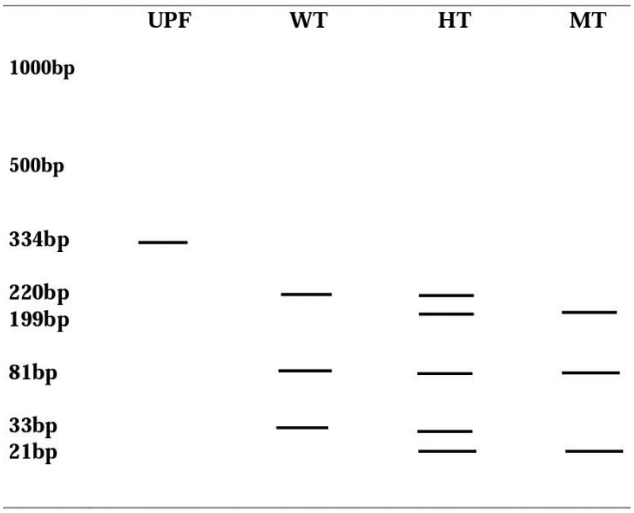

Figure 3. 2: A schematic diagram of PCR-RFLP electrophoregram of *CYP3A4*1B (-290 A>G)* alleles.

Where UPF is undigested PCR product, WT is Wild-type (A), HT is heterozygous (A/G) and MT (G) is mutant.

3.9.3 Identification of *MDR1_Ex12 (1236 C>T)* genotypes by PCR-RFLP

PCR for *MDR1_Ex12 (1236 C>T)* gene was done in a 20µL total reaction volume. The reaction mixture was made up of nuclease-free water, 10µM forward (F) primer, reverse (R) primer, One Taq Quick-Load 2X Master Mix Standard Buffer and genomic DNA. The details of this PCR mixture and PCR cycling conditions are contained in Tables 3.7 and Table 3.8 respectively. A negative control reaction was prepared that contained all other reagents except genomic DNA.

Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 5µL of the PCR products into each well in the agarose

gel. The setup was electrophoresed at 75V for 45minutes and then visualized with a gel imager. The expected PCR product size is 366bp.

Aliquots of the PCR products were digested with appropriate restriction enzyme (Hae III) in a 20µL reaction mix containing nuclease-free water, 10X Buffer R and Hae III restriction enzyme as shown in Table 3.9. The reaction mixture was prepared in a sterile Eppendorf tube and incubated for 3 hours at 37^{O}C. Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 10µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 85V for 60minutes and then visualized with a gel imager.

Expected fragments sizes represented in fig. 3.2 are 269bp, 62bp and 35bp for wild-type allele; 269bp, 97bp, 62bp and 35bp for heterozygous, 269 and 97bp for mutant allele.

Table 3. 7: *MDR1_Ex12 (1236 C>T)* gene PCR reaction mixture

Reagents	**Volume (µL)**
Nuclease-free water	1
Forward primer	2
Reverse primer	2
Master Mix	10
Genomic DNA	5
Total	20

Table 3. 8: *MDR1_Ex12 (1236 C>T)* gene PCR cycling conditions

Conditions	**Temperature/^{O}C**	**Time**	**Number of Cycles**
Initial denaturation	94	2min	1
Denaturation	94	30sec	35
Annealing	60	30sec	
Extension	72	30sec	
Final extension	72	7min	1

Table 3. 9: Restriction digest reaction mixture for *MDR1_Ex12 (1236 C>T)* gene

Reagent	**Volume (µL)**
Nuclease-free water	7
10X buffer R	2
Hae III	1
PCR product	10
Total	20

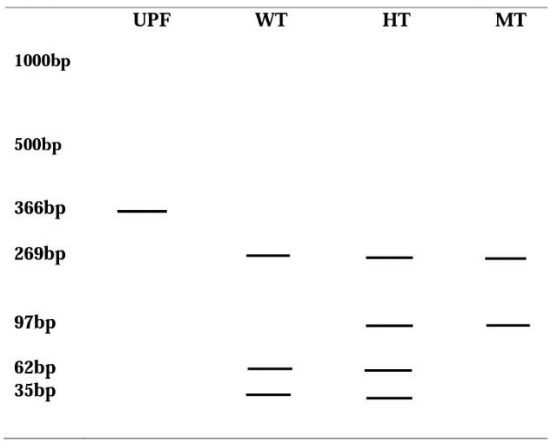

Figure 3. 3: A schematic diagram of PCR-RFLP electrophoregram of *MDR1_Ex12 (1236 C>T)* alleles.

Where UPF is undigested PCR product, WT is Wild-type (C), HT is heterozygous (C/T) and MT (T) is mutant.

3.9.4 Identification of *MDR1_Ex21 (2677 G>A)* genotypes by PCR-RFLP

PCR for *MDR1_Ex21 (2677 G>A)* gene was done in a 20µL total reaction volume. The reaction mixture was made up of nuclease-free water, 10µM forward (F) primer, reverse (R) primer, One Taq Quick-Load 2X Master Mix Standard Buffer and genomic DNA. The details of this PCR mixture and PCR cycling conditions are contained in Table 3.10 and Table 3.11 respectively. A negative control reaction was prepared that contained all other reagents except genomic DNA.

Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA (5µL) ladder was loaded against 5µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 75V for 45minutes and then visualized with a gel imager. The expected PCR product size is 220bp.

Aliquots of the PCR products were digested with appropriate restriction enzyme (Bsr I) in a 20µL reaction mix containing nuclease-free water, 10X Buffer B and Bsr I restriction enzyme as shown in Table 3.12. The reaction mixture was prepared in a sterile Eppendorf tube and incubated for 3 hours at 37^{O}C. Agarose gel (3% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA (5µL) was loaded against 10µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 85V for 60minutes and then visualized with a gel imager.

Expected fragments sizes represented in fig. 3.4 are 220bp for wild-type allele; 220bp, 206bp and 14bp for heterozygous; 206 and 14bp for mutant allele.

Table 3. 10: *MDR1_Ex21 (2677 G>A)* gene PCR reaction mixture

Reagents	**Volume (µL)**
Nuclease-free water	1
Forward primer	2
Reverse primer	2
Master Mix	10
Genomic DNA	5
Total	20

Table 3. 11: *MDR1_Ex21 (2677 G>A)* gene PCR cycling conditions

Conditions	**Temperature/^{O}C**	**Time**	**Number of Cycles**
Initial denaturation	94	2min	1
Denaturation	94	30sec	35
Annealing	60	30sec	
Extension	72	30sec	
Final extension	72	7min	1

Table 3. 12: Restriction digest reaction mixture for *MDR1_Ex21 (2677 G>A)* gene

Reagents	**Volume (µL)**
Nuclease-free water	7
10X buffer B	2
Bsr I	1
PCR product	10
Total	20

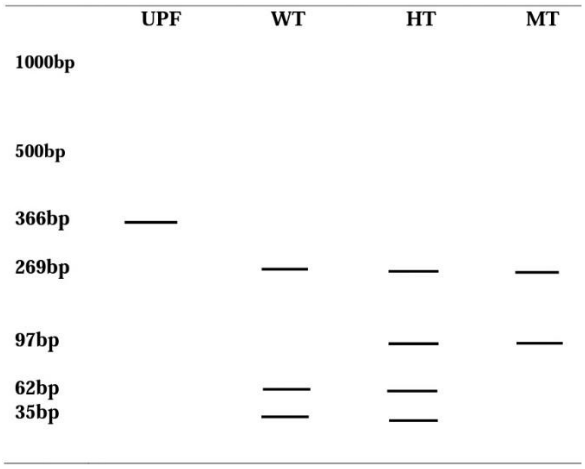

Figure 3. 4: A schematic diagram of PCR-RFLP electrophoregram of *MDR1_Ex21 (2677 G>A)* alleles.

Where UPF is undigested PCR product, WT is Wild-type (G), HT is heterozygous (G/A) and MT (A) is mutant.

3.9.5 Identification of *MDR1_Ex21 (2677 G>T)* genotypes by PCR-RFLP

PCR for *MDR1_Ex21 (2677 G>T)* gene was done in a 20µL total reaction volume. The reaction mixture was made up of nuclease-free water, 10µM forward (F) primer, reverse (R) primer, One Taq Quick-Load 2X Master Mix Standard Buffer and genomic DNA. The details of this PCR mixture and PCR cycling conditions are contained in Table 3.13 and Table 3.14, respectively. A negative control reaction was prepared that contained all other reagents except genomic DNA.

Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 5µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 75V for 45minutes and then visualized with a gel imager. The expected PCR product size is 224bp.

Aliquots of the PCR products were digested with appropriate restriction enzyme (Ban I) in a 20µL reaction mix containing nuclease-free water, 10X Buffer O and Ban I restriction enzyme as shown in Table 3.15. The reaction mixture was prepared in a sterile Eppendorf tube and incubated for 3 hours at 37^{O}C. Agarose gel (3% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA ladder (5µL) was loaded against 10µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 85V for 60minutes and then visualized with a gel imager.

Expected fragments sizes represented in fig. 3.5 are 224bp for wild-type allele; 224bp, 198bp and 28bp for heterozygous; 198 and 28bp for mutant allele.

Table 3. 13: *MDR1_Ex21 (2677 G>T)* gene PCR reaction mixture

Reagents	**Volume (µL)**
Nuclease-free water	1
Forward primer	2
Reverse primer	2
Master Mix	10
Genomic DNA	5
Total	20

Table 3. 14: *MDR1_Ex21 (2677 G>T)* gene PCR cycling conditions

Conditions	**Temperature/^{O}C**	**Time**	**Number of Cycles**
Initial denaturation	94	2min	1
Denaturation	94	30sec	35
Annealing	60	30sec	
Extension	72	30sec	
Final extension	72	7min	1

Table 3. 15: Restriction digest reaction mixture for *MDR1_Ex21 (2677 G>T)* gene

Reagent	**Volume (µL)**
Nuclease-free water	7
10X buffer O	2
Ban I	1
PCR product	10
Total	20

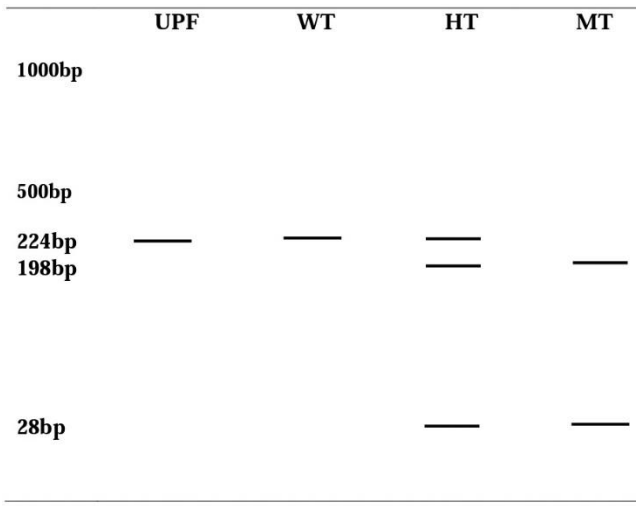

Figure 3. 5: A schematic diagram of PCR-RFLP electrophoregram of *MDR1_Ex21 (2677 G>T)* alleles.

Where UPF is undigested PCR product, WT is Wild-type (G), HT is heterozygous (G/T) and MT (T) is mutant.

3.9.6 Identification of *MDR1_Ex26 (3435 C>T)* genotypes by PCR-RFLP

PCR for *MDR1_Ex26 (3435 C>T)* gene was done in a 20µL total reaction volume. The reaction mixture was made up of nuclease-free water, 10µM forward (F) primer, reverse (R) primer, One Taq Quick-Load 2X Master Mix Standard Buffer and genomic DNA. The details of this PCR mixture and PCR cycling conditions are contained in Table 3.16 and Table 3.17, respectively. A negative control reaction was prepared that contained all other reagents except genomic DNA.

Agarose gel (2% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA (5µL) ladder was loaded against 5µL of the PCR products into each well in the agarose

gel. The setup was electrophoresed at 75V for 45minutes and then visualized with a gel imager. The expected PCR product size is 248bp.

Aliquots of the PCR products were digested with appropriate restriction enzyme (Mbo I) in a 20µL reaction mix containing nuclease-free water, 10X Buffer R and Mbo I restriction enzyme as shown in Table 3.18. The reaction mixture was prepared in a sterile Eppendorf tube and incubated for 3 hours at 37^{O}C. Agarose gel (3% w/v) stained with 3µL ethidium bromide was cast in a gel trough, allowed to solidify and immersed in the 1X Tris-acetate-EDTA (TAE) buffer contained in a gel tank. A 100 bp DNA (5µL) was loaded against 10µL of the PCR products into each well in the agarose gel. The setup was electrophoresed at 85V for 60minutes and then visualized with a gel imager.

Expected fragments sizes represented in fig. 3.6 are 172bp, 60bp and 16bp for wild-type allele; 232bp, 172bp, 60bp and 16bp for heterozygous; 232 and 16bp for mutant allele.

Table 3. 16: *MDR1_Ex26 (3435 C>T)* gene PCR reaction solution

Reagents	**Volume (µL)**
Nuclease-free water	1
Forward primer	2
Reverse primer	2
Master Mix	10
Genomic DNA	5
Total	20

Table 3. 17: *MDR1_Ex26 (3435 C>T)* gene PCR cycling conditions

Conditions	**Temperature/^{O}C**	**Time**	**Number of Cycles**
Initial denaturation	94	2min	1
Denaturation	94	30sec	35
Annealing	60	30sec	
Extension	72	30sec	
Final extension	72	7min	1

Table 3. 18: Restriction digest reaction solution for *MDR1_Ex26 (3435 C>T)* gene

Reagents	**Volume (µL)**
Nuclease-free water	7
10X buffer R	2
Mbo I	1
PCR product	10
Total	20

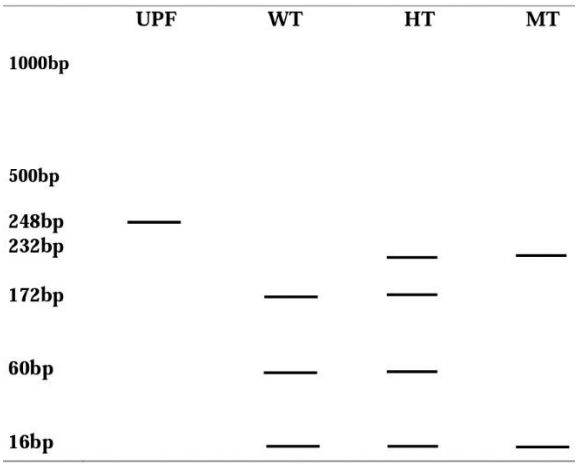

Figure 3. 6: A schematic diagram of PCR-RFLP electrophoregram of *MDR1_Ex26 (3435 C>T)* alleles.

Where UPF is undigested PCR product, WT is Wild-type (C), HT is heterozygous (C/T) and MT (T) is mutant.

Statistical Analysis

Data obtained from both questionnaire administration and laboratory genotyping were entered into Microsoft Access 2013 table and analyzed. The data was summarized as frequencies and proportions. Chi-squared test ($p^2 + 2pq + q^2 = 1$) was used to determine whether the genotype distribution is consistent with Hardy-Weinberg equilibrium. Given the homozygous wild-type genotype (AA), heterozygous carrier (Aa) and homozygous variant (aa), p is the frequency of 'A' allele, q is the frequency of 'a' allele.

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of study participants

A total of 87 patients were recruited into this study, 82 (94.25%) were ESRD patients on dialysis management and 5 (5.76%) were kidney transplant patients who were previously suffering from ESRD. Among the patients, 62 (71.26%) were males whiles 25 (28.74%) were females. The average ages of all recruited patients were 46 years ±14.39 years. Among the patient, 29 (33.33%) were within the ages 21-40 years, 38 (43.68%) within the age range of 41-60 years and 20 (22.99%) were above 60 years. From the data collected it was observed that 46 (52.87%) of the patients were actively working and 41 (47.13%) were unemployed. The characteristics of the study participants are shown in Table 4.1.

Table 4. 1: Socio-Demographic data

Characteristics		**n**	**Frequency (%)**
Age (years)	(Mean ± SD) = 46±14.39		
		N=87	
	1-20	0	0
	21-40	29	33.33
	41-60	38	43.68
	61 and above	20	22.99
Sex	Male	62	71.26
	Female	25	28.74
Marital Status	Married	55	63.22
	Single	28	32.18
	Divorced	2	2.30
	Widow	2	2.30
Employment status	Employed	46	52.87
	Unemployed	41	47.13

N; Total number of patients, n; number of patients within a group, frequency of patients, SD; Standard deviation.

4.2: Clinical History of study participants

All the patients recruited into the study were previously suffering from a disease condition that deteriorated their kidneys causing ESRD. Among the patients, 86 (98.85%) had Hypertension, 7 (8.05%) had Diabetes, 2 (2.30%) had Sickle Cell disease and 2 (2.30%) had Systemic Lupus Erythematosus. The clinical history of study participants is shown in Table 4.2

Table 4. 2: Clinical History

Characteristics		**n**	**Frequency (%)**
		N=87	
Etiology	Yes	87	100
	No	0	0.00
Stages of Renal Impairment	ESRD (under Dialysis)	82	94.25
	Transplant	5	5.76
	Hypertension	86	98.85
	Diabetes	7	8.05
	Sickle Cell	2	2.30
	Systemic Lupus Erythematosus	2	2.30
	Nephrotic Syndrome	0	0.00
	Chronic Glomerulonephritis	0	0.00
	Kidney biopsy	0	0.00

N; Total number of patients, n; number of patients within a group, Percentage frequency and total frequency may add to >100 and >87 since many patients suffered from more than one co-morbidity.

4.3: Characteristics of medication used by study participants

Out of 82 dialysis participants recruited into the study, only 69 (84.15%) of them are on medication alongside the dialysis and or transplant treatment, and the rest do not take any medication. The data collected showed that most of the patients take anti-hypertensive. The data also showed that 46 (56.10%) of the patients use analgesics without their medical providers prescribing it.

Table 4. 3: Medication used by study participants

Medication		n	Frequency (%)
		N=82	
Medication Use	Yes	69	84.15
	No	13	15.85
Antihypertensive	Nifedipine	21	25.61
	Amlodipine	24	32.93
	Atenolol	4	4.88
	Bisoprolol	18	21.95
	Metoprolol	2	2.44
	Lisinopril	6	7.32
	Captopril	2	2.44
	Carvedilol	6	7.32
	Methyldopa	25	30.49
Diuretic	Furosemide	12	14.63
	Spironolactone	1	1.22
Miscellaneous	Prednisolone	5	6.10
	Iron replacement	6	7.32
	Calcium carbonate	2	2.44
	Vancomycin	1	1.22
	Calcium Resonance	1	1.22
	Hydralazine	1	1.22
	Erythropoietin	1	1.22
	Omeprazole	4	4.88
	Cefuroxime	1	1.22
	Tramadol	1	1.22
	Mycophenolate mofitel	2	2.44
	Azithromycin	3	3.66
	Ranitidine	9	10.98
	Folic acid	1	1.22
Use of Analgesics	Yes	46	56.10
	No	36	43.90
Types of Analgesics	Paracetamol	41	89.13
	Diclodec	2	4.35
	Aspirin	1	2.17
	Ibuprofen	3	6.52

N; Total number of patients, n; frequency of patients, Percentage frequency may add to >100 since many patients use medications belonging to different groups.

4.4 Genotypic profile

4.4.1: Identification of *CYP3A5*3 (6986 A>G)* genotypes by PCR-RFLP

The *CYP3A5*3 (6986 A>G)* alleles and genotypes were identified by the PCR-RFLP technique. The PCR products of *CYP3A5*3 (6986 A>G)* primer amplifications were taken through gel electrophoresis by loading the samples on to a 2% agarose gel stained with ethidium bromide. The samples were loaded together with a 100bp DNA ladder, a positive control (500bp PCR product) and a negative control (PCR solution without DNA). Electrophoresis result was viewed with a UV Gel Imager. Figure 4.1 shows the electrophoresis result before the samples were digested with the Ssp I restriction enzymes. The digested products were run on a 3% agarose gel electrophoresis. This is shown in figure 4.2.

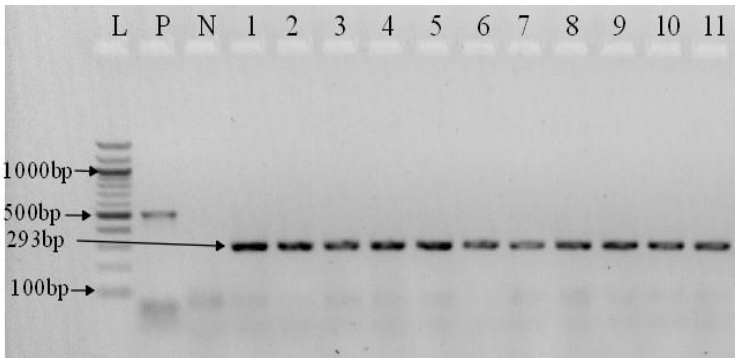

Figure 4. 1: *CYP3A5*3 (6986 A>G)* PCR amplicon Gel electrophoregram

A 2% gel electrophoregram showing a 293 bp positions of DNA after PCR amplification with *CYP3A5*3 (6986 A>G)* forward and reverse primers. L: 100bp DNA ladder, P: Positive control, N: Negative control, 1 – 11: PCR products.

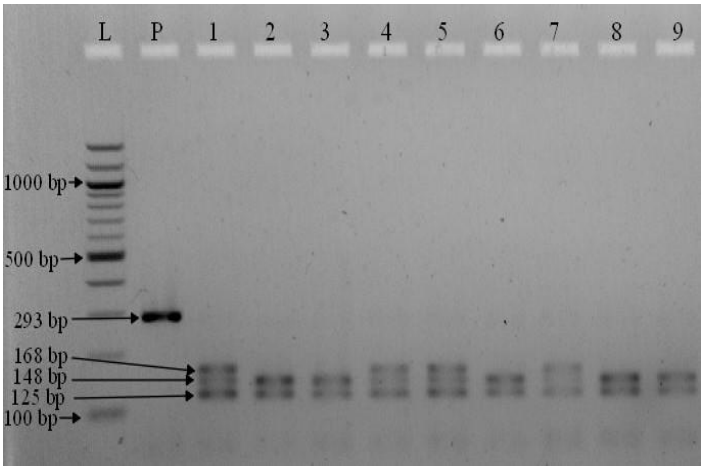

Figure 4. 2: *CYP3A5*3* (6986 A>G) PCR-RFLP Gel electrophoregram

A 3% agarose gel electrophoregram showing RFLP results for *CYP3A5*3 (6986 A>G)* PCR product digested using Ssp I restriction enzymes. L-100bp DNA ladder, P: Undigested fragment, 1-9: Digested samples.

Among the 87 study participants who were genotyped, 63 (72.40%) of them expressed wild-type genotype AA, 20 (23%) expressed the heterozygous genotype AG and 4 (4.6%) expressed the mutant genotype GG of the *CYP3A5*3 (6986 A>G)* genes. Among the 82 dialysis patients, 58 (70.73%) expressed wild-type the genotype AA, 20 (24.39%) expressed the heterozygous genotype AG and 4 (4.88%) expressed the mutant genotype GG. All transplants patients expressed the wild-type genotype AA. The allele and genotypic frequencies of study participants is shown in Table 4.4.

Table 4. 4: Allele and Genotypic frequencies of *CYP3A5*3 (6986 A>G)* gene

	Allele	**n**	**Frequency (%)**	**Genotype**	**n**	**Frequency (%)**
All		**N=174**			**N=87**	
	A	146	83.91	AA	63	72.40
	G	28	16.09	AG	20	23.00
				GG	4	4.60
Dialysis		**N=164**			**N=82**	
	A	136	82.93	AA	58	70.73
	G	28	17.07	AG	20	24.39
				GG	4	4.88
Transplant		**N=10**			**N=5**	
	A	10	100	AA	5	100
	G	0	0	AG	0	0
				GG	0	0

n; number per group , N; Total number, A; Wild-type allele, G; Variant allele. AA= Homozygous wild-type, AG= Heterozygous, GG= Homozygous mutant.

Hardy-Weinberg analysis of *CYP3A5*3 (6986 A>G)* gene

The sum of the entries $p^2 + 2pq + q^2$ should be equal to 1

$(0.724)^2 + (2\times0.23) + (0.04)^2 = 0.5242 + 0.46 + 0.0016 = 0.9858$

4.4.2: Identification *CYP3A4*1B (-290 A>G)* genotypes by PCR-RFLP

The *CYP3A4*1B (-290 A>G)* alleles and genotypes were identified by the PCR-RFLP technique. The PCR products of *CYP3A4*1B (-290 A>G)* primer amplifications were taken through gel electrophoresis by loading the samples on to a 2% agarose gel stained with ethidium bromide. The samples were loaded together with a 100bp DNA ladder, a positive control (500bp PCR product) and a negative control (PCR solution without DNA). Electrophoresis result was viewed with a UV Gel Imager. Figure 4.3 shows the electrophoresis result before the samples were digested with the Pst I restriction enzymes. The digested products were run on a 3% agarose gel electrophoresis. This is shown in figure 4.4

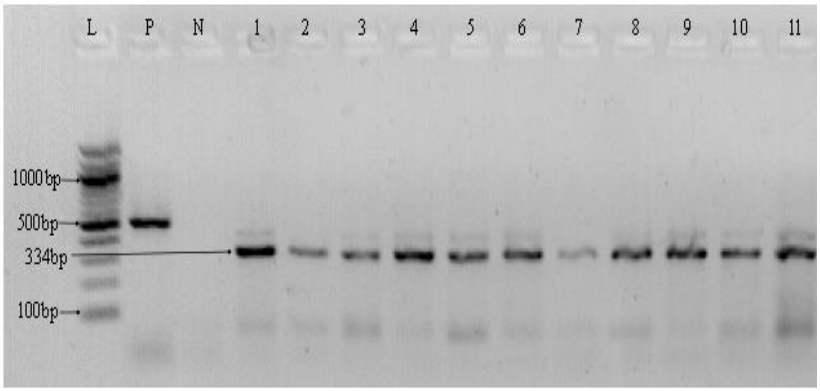

Figure 4. 3: *CYP3A4*1B (-290 A>G)* PCR amplicon Gel electrophoregram

A 2% gel electrophoregram showing a 334 bp positions of DNA after PCR amplification with *CYP3A4*1B (-290 A>G)* forward and reverse primers. L: 100bp DNA ladder, P: Positive control, N: Negative control, 1 – 11: PCR products.

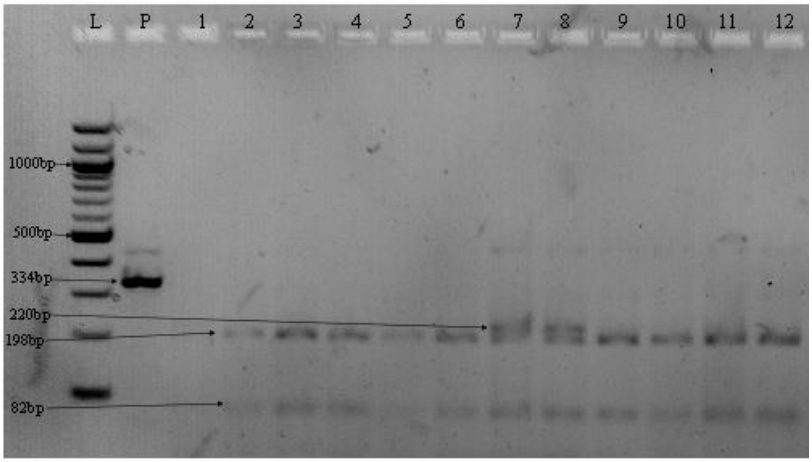

Figure 4. 4: *CYP3A4*1B (-290 A>G)* PCR-RFLP Gel electrophoregram

A 3% agarose gel electrophoregram showing RFLP results for *CYP3A4*1B (-290 A>G)* PCR product digested using Pst I restriction enzymes. L-100bp DNA ladder, P: Undigested fragment, 1-12: Digested samples.

Among the study participants genotyped for *CYP3A4*1B (-290 A>G)* genes, 6 (6.90%) of them expressed the wild-type genotype AA, 13.79% expressed the heterozygous genotype AG and 69 (79.31%) expressed the mutant genotype GG. Among the 82 dialysis patients, 6 (7.32%) expressed the wild-type genotype AA, 11 (13.41%) expressed the heterozygous genotype AG and 65 (79.27%) expressed the mutant genotype GG. Among the transplant patients, 4 (80%) of them expressed the mutant genotype GG and 1 (20%) expressed the heterozygous genotype AG. None of the transplant patients expressed the wild-type genotype AA. This is shown in Table 4.5

Table 4. 5: Allele and Genotypic frequencies of *CYP3A4*1B (-290 A>G)* gene

	Allele	**n**	**Frequency (%)**	**Genotype**	**n**	**Frequency (%)**
All		**N=174**			**N=87**	
	A	24	13.79	AA	6	6.90
	G	150	86.21	AG	12	13.79
				GG	69	79.31
Dialysis		**N=164**			**N=82**	
	A	23	14.02	AA	6	7.32
	G	141	85.98	AG	11	13.41
				GG	65	79.27
Transplant		**N=10**			**N=5**	
	A	1	10	AA	0	0
	G	9	90	AG	1	20
				GG	4	80

n; number in a group, N; Total number, A; Wild-type allele, G; Variant allele. AA= Homozygous wild-type, AG= Heterozygous, GG= Homozygous mutant.

Hardy-Weinberg analysis of *CYP3A4*1B (-290 A>G) gene*

The sum of the entries $p^2+2pq+ q^2$ should be equal to 1

$(0.069)^2 + (2\times0.1379) + (0.7931)^2 = 0.004761 + 0.2758 + 0.62901 = 0.91$

4.4.3: Identification *MDR1_Ex12 (1236 C>T)* genotypes by PCR-RFLP

The *MDR1_Ex12 (1236 C>T)* alleles and genotypes were identified by the PCR-RFLP technique. The PCR products of *MDR1_Ex12 (1236 C>T)* primer amplifications were taken through gel electrophoresis by loading the samples on to a 2% agarose gel stained with ethidium bromide. The samples were loaded together with a 100bp DNA ladder, a positive control (500bp PCR product) and a negative control (PCR solution without DNA). Electrophoresis result was viewed with a UV Gel Imager. Figure 4.5 shows the electrophoresis result before the samples were digested with the Hae III restriction enzymes. The digested products were ran on a 2% agarose gel electrophoresis. This is shown in figure 4.6.

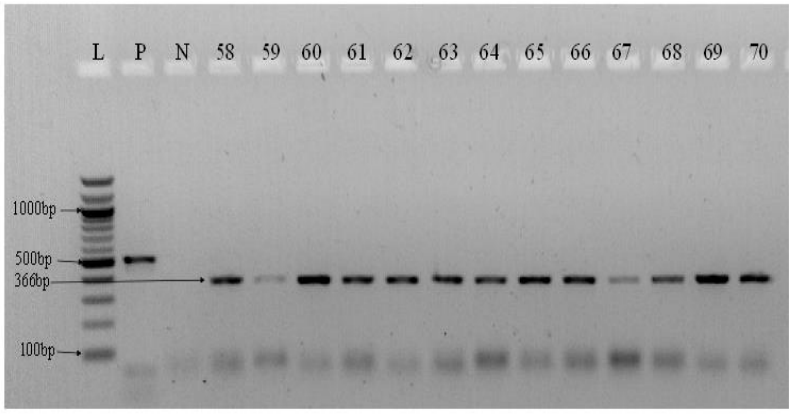

Figure 4. 5: *MDR1_Ex12 (1236 C>T)* PCR amplicon Gel electrophoregram

A 2% gel electrophoregram showing a 366 bp positions of DNA after PCR amplification with *MDR1_Ex12 (1236 C>T)* forward and reverse primers. L: 100bp DNA ladder, P: Positive control, N: Negative control, 58-70: PCR products.

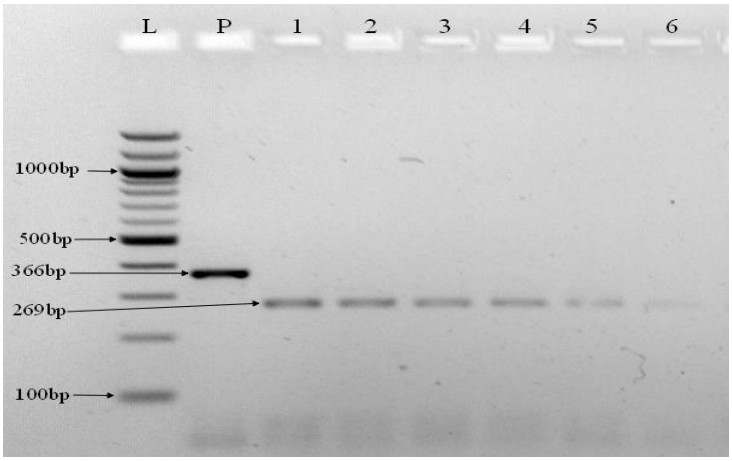

Figure 4. 6*: MDR1_Ex12 (1236 C>T)* RFLP-PCR Gel electrophoregram

A 2% agarose gel electrophoregram showing RFLP results for *MDR1_Ex12 (1236 C>T)* PCR product digested using Hae III restriction enzymes. L-100bp DNA ladder, P: Undigested fragment, 1-6: Digested samples.

All of the study participants expressed the wild-type genotype CC of the *MDR1_Ex12 (1236 C>T)* gene. None of them expressed the heterozygous and mutant genes. This is shown in Table 4.6

Table 4. 6: Allele and Genotypic frequencies of *MDR1_Ex12 (1236 C>T)* gene

	Allele	**n**	**Frequency (%)**	**Genotype**	**n**	**Frequency (%)**
All		**N=174**			**N=87**	
	C	174	100	CC	87	100
	T	0	0	CT	0	0
				TT	0	0
Dialysis		**N=164**			**N=82**	
	C	164	100	CC	82	100
	T	0	0	CT	0	0
				TT	0	0
Transplant		**N=10**			**N=5**	
	C	10	100	CC	5	100
	T	0	0	CT	0	0
				TT	0	0

n; number per group, N; Total number, C; Wild-type allele, T; Variant allele. CC= Homozygous wild-type, CT= Heterozygous, TT= Homozygous mutant

Hardy-Weinberg analysis of *MDR1_Ex12 (1236 C>T)* gene

The sum of the entries $p^2+2pq+q^2$ should be equal to 1

$(1)^2 + (2\times0) + (0)^2 = 1$

4.4.4: Identification *MDR1_Ex21 (2677 G>A)* genotypes by PCR-RFLP

The *MDR1_Ex21 (2677 G>A)* alleles and genotypes were identified by the PCR-RFLP technique. The PCR products of *MDR1_Ex21 (2677 G>A)* primer amplifications were taken through gel electrophoresis by loading the samples n to a 2% agarose gel stained with ethidium bromide. The samples were loaded together with a 100bp DNA ladder, a positive control (500bp PCR product) and a negative control (PCR solution without DNA). Electrophoresis result was viewed with a UV Gel Imager. Figure 4.7 shows the electrophoresis result before the samples were digested with the Bsr I restriction enzymes. The digested products were run on a 2% agarose gel electrophoresis. This is shown in figure 4.8.

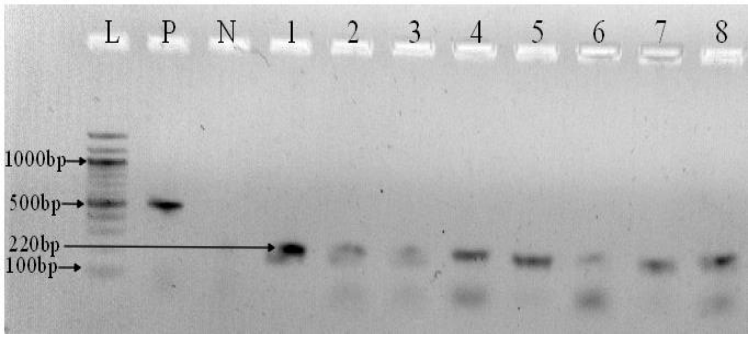

Figure 4. 7: *MDR_Ex21 (2677 G>A)* PCR amplicon Gel electrophoregram

A 2% gel electrophoregram showing a 220 bp positions of DNA after PCR amplification with *MDR1_Ex21 (2677 G>A)* forward and reverse primers. L: 100bp DNA ladder, P: Positive control, N: Negative control, 1-8: PCR products.

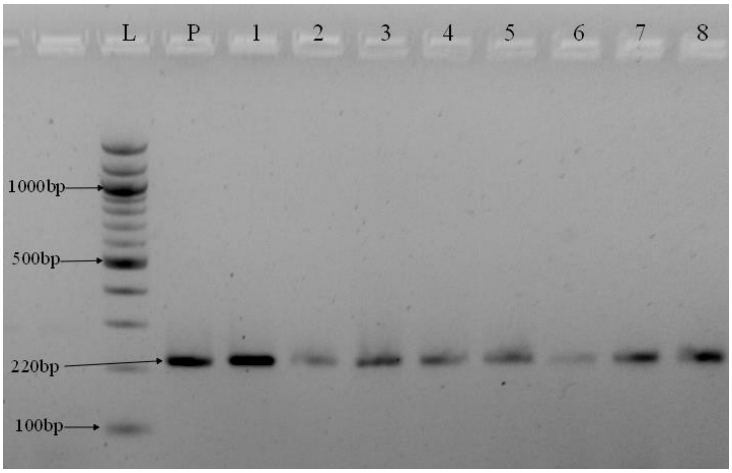

Figure 4. 8*: MDR1_Ex21 (2677 G>A)* PCR-RFLP Gel electrophoregram

A 2% agarose gel electrophoregram showing RFLP results for *MDR1_Ex21 (2677 G>A)* PCR product digested using Bsr I restriction enzymes. L-100bp DNA ladder, P: Undigested fragment, 1-8: Digested samples.

All of the study participants expressed the wild-type genotype GG of the *MDR1_Ex21 (2677 G>A)* gene. None of them expressed the heterozygous and mutant genotypes. This is shown in Table 4.7.

Table 4. 7: Allele and Genotypic frequencies of *MDR1_Ex21 (2677 G>A)*

	Allele	n	Frequency (%)	Genotype	n	Frequency (%)
All		**N=174**			**N=87**	
	G	174	100	GG	87	100
	A	0	0	GA	0	0
				AA	0	0
Dialysis		**N=164**			**N=82**	
	G	164	100	GG	82	100
	A	0	0	GA	0	0
				AA	0	0
Transplant		**N=10**			**N=5**	
	G	10	100	GG	5	100
	A	0	0	GA	0	0
				AA	0	0

n; number in a group, N; Total number, G; Wild-type allele, A; Variant allele. GG= Homozygous wild-type, GA= Heterozygous, AA= Homozygous mutant

Hardy-Weinberg analysis of *MDR1_Ex 21(2677 G>A)* gene

The sum of the entries $p^2+2pq+q^2$ should be equal to 1

$(1)^2 + (2\times0) + (0)^2 = 1$

4.4.5: Identification *MDR1_Ex21 (2677 G>T)* genotypes by PCR-RFLP

The *MDR1_Ex21 (2677 G>T)* alleles and genotypes were identified by the PCR-RFLP technique. The PCR products of *MDR1_Ex21 (2677 G>T)* primer amplifications were taken through gel electrophoresis by loading samples on to a 2% agarose gel stained with ethidium bromide. The samples were loaded together with a 100bp DNA ladder, a positive control (500bp PCR product) and a negative control (PCR solution without DNA). Electrophoresis result was viewed with a UV Gel Imager. Figure 4.9 shows the electrophoresis result before the samples were digested with the Ban I restriction enzymes. The digested products were run on a 2% agarose gel electrophoresis. This is shown in figure 4.10

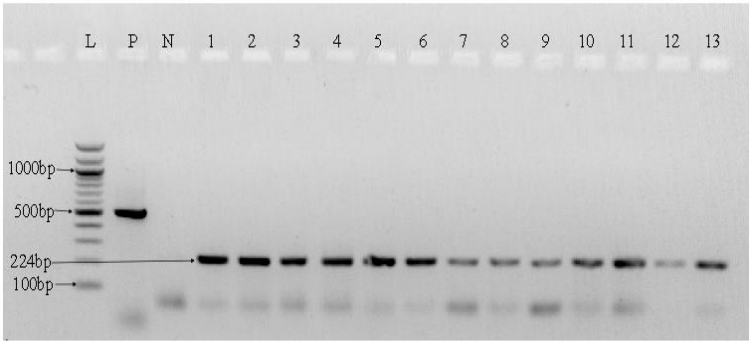

Figure 4. 9: *MDR1_Ex21 (2677 G>T)* PCR amplicon Gel electrophoregram

A 2% gel electrophoregram showing a 224 bp positions of DNA after PCR amplification with *MDR1_Ex21 (2677 G>T)* forward and reverse primers. L: 100bp DNA ladder, P: Positive control, N: Negative control, 1 – 13: PCR products.

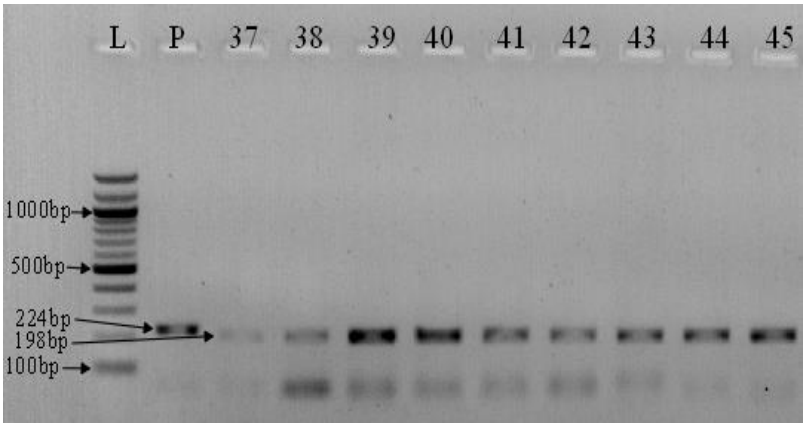

Figure 4. 10: *MDR1_Ex21 (2677 G>T)* PCR-RFLP Gel electrophoregram

A 2% agarose gel electrophoregram showing RFLP results for *MDR1_Ex21 (2677 G>T)* PCR product digested using Ban I restriction enzymes. L-100bp DNA ladder, P: Undigested fragment, 37-45: Digested samples.

The data obtained showed that 86 (98.85%) of the study participants expressed the wild-type genotype GG, 1 (1.15%) expressed the heterozygous genotype GT and none of them expressed the mutant genotype TT. Among the 82 dialysis patients, 81 (98.78%) expressed the wild-type gene, whilst the rest expressed the heterozygous gene. All transplant patients genotyped expressed the wild-type genotype GG. This is shown in Table 4.8.

Table 4. 8: Allele and Genotypic frequencies of *MDR1_Ex21 (2677 G>T)*

	Allele	n	Frequency (%)	Genotype	n	Frequency (%)
All		**N=174**			**N=87**	
	G	173	99.43	GG	86	98.85
	T	1	0.57	GT	1	1.15
				TT	0	0
Dialysis		**N=164**			**N=82**	
	G	163	99.39	GG	81	98.78
	T	1	0.61	GT	1	1.22
				TT	0	
Transplant		**N=10**			**N=5**	
	G	10	100	GG	5	100
	T	0	0	GT	0	0
				TT	0	0

n; number in a group, N; Total number, G; Wild-type allele, T; Variant allele. GG= Homozygous wild-type, GT= Heterozygous, TT= Homozygous mutant

Hardy-Weinberg analysis of *MDR1_Ex21 (2677 G>T)* gene

The sum of the entries $p^2+2pq+q^2$ should be equal to 1

$(0.9885)^2 + (2\times0.0115) + 0 = 0.9771+ 0.023 = 1$

4.8 Identification *MDR1_Ex26 (3435 C>T)* genotypes by PCR-RFLP

The *MDR1_Ex26 (3435 C>T)* alleles and genotypes were identified by the PCR-RFLP technique. The PCR products of *MDR1_Ex26 (3435 C>T)* primer amplifications were taken through gel electrophoresis by loading the samples on to a 2% agarose gel stained with ethidium bromide. The samples were loaded together with a 100bp DNA ladder, a positive control (500bp PCR product) and a negative control (PCR solution without DNA). Electrophoresis result was viewed with a UV Gel Imager. Figure 4.11 shows the electrophoresis result before the samples were digested with the Mbo I restriction enzymes. The digested products were run on a 3% agarose gel electrophoresis. This is shown in figure 4.12.

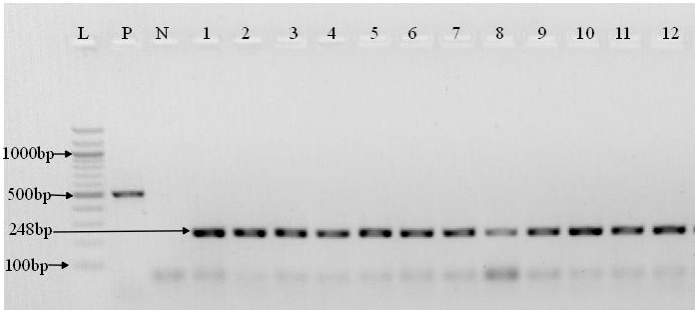

Figure 4. 11: *MDR1_Ex26 (3435 C>T)* PCR amplicon Gel electrophoregram

A 2% gel electrophoregram showing a 248 bp positions of DNA after PCR amplification with *MDR1_Ex26 (3435 C>T)* forward and reverse primers. L: 100bp DNA ladder, P: Positive control, N: Negative control, 1 – 12: PCR products.

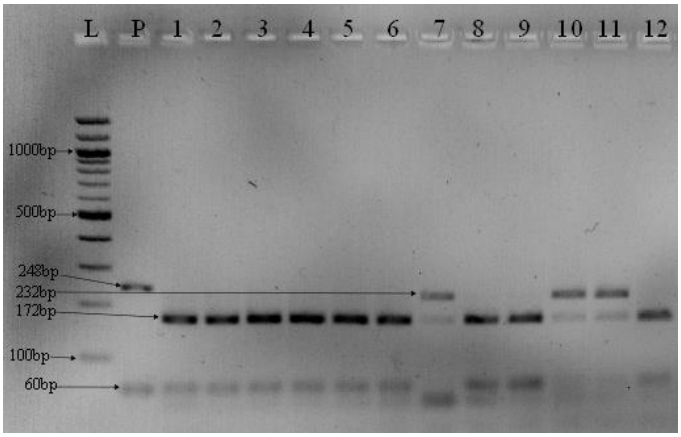

Figure 4. 12: *MDR1_Ex26 (3435 C>T)* PCR-RFLP Gel electrophoregram

A 3% agarose gel electrophoregram showing RFLP results for *MDR1_Ex26 (3435 C>T)* PCR product digested using Ban I restriction enzymes. L-100bp DNA ladder, P: Undigested fragment, 1-12: Digested samples.

The data obtained showed that 65 (74.71%) of the study participants expressed the wild-type genotype CC, 21 (24.14%) expressed the heterozygous genotype CT and 1 (1.15%) expressed the mutant genotype TT. Among the 82 dialysis patients, 63 (76.83%) expressed the wild-type genotype CC, 18 (21.95%) expressed the heterozygous genotype CT and only 1 (1.22%) expressed the mutant genotype TT. Among the transplant patients, 2 (40%) expressed the wild-type genotype CC and 3 (60%) expressed the heterozygous genotype CT. None of the transplant patients expressed the mutant genotype. This is shown in Table 4.9.

Table 4. 9: Allele and Genotypic frequencies of *MDR1_Ex26 (3435 C>T)*

	Allele	n	Frequency (%)	Genotype	n	Frequency (%)
All		**N=174**			**N=87**	
	C	151	86.78	CC	65	74.71
	T	23	13.22	CT	21	24.14
				TT	1	1.15
Dialysis		**N=164**			**N=82**	
	C	144	87.80	CC	63	76.83
	T	20	12.20	CT	18	21.95
				TT	1	1.22
Transplant		**N=10**			**N=5**	
	C	7	70	CC	2	40
	T	3	30	CT	3	60
				TT	0	0

n; number in a group, N; Total number, C; Wild-type allele, T; Variant allele. CC= Homozygous wild-type, CT= Heterozygous, TT= Homozygous mutant

Hardy-Weinberg analysis of *MDR1_Ex26 (3435 C>T)* gene

The sum of the entries $p^2+2pq+q^2$ should be equal to 1

$(0.7471)^2+ (2\times0.2414) + (0.0115)^2= 0.5581+0.4828+0.0001 =1.04$

4.6: Distribution between Etiology and genotypic profile

4.6.1 Distribution between etiology and *CYP3A5*3 (6986 A>G)* genes.

The data shows that the ESRD conditions of the study participants were caused primarily by diabetes and hypertension. Among them, 7 suffered from diabetes and 86 suffered from hypertension. Among the 7 patients who had diabetes, 57.14% expressed the wild-type genotype AA and the rest expressed the heterozygous genotype AG. None of them expressed the mutant genotype GG. Also, among the 86 whose ESRD was caused by hypertension, 72.09% expressed the wild-type genotype AA, 23.26% expressed the heterozygous genotype AG and 4.65% expressed the mutant genes.

Table 4. 10: Distribution between Co-morbidity and the genotypic profile

Etiology		**N=87**	**Total Frequency (%)**	***CYP3A5*3* Frequency (%)**		
		n		AA	AG	**GG**
Diabetes	**Present**	7	8.05	57.14	42.86	0
	Absent	80	91.95	73.75	21.25	5
HBP	**Present**	86	98.85	72.09	23.26	4.65
	Absent	1	1.15	100	0	0

N, Total number of patients, n; number of patients in a group, HBP; High Blood Pressure, A; Wild-type allele, G; Variant allele. AA= Homozygous wild-type, AG= Heterozygous, GG= Homozygous mutant.

4.6.2: Distribution between etiology and *CYP3A4*1B (-290 A>G)* genes.

The data shows that the ESRD conditions of the study participants were caused primarily by diabetes and hypertension. Among them, 7 suffered from diabetes and 86 suffered from hypertension. Among the 7 patients who had diabetes, 14.29% expressed the wild-type genotype AA and 85.71% expressed the mutant genotype GG. None of them expressed the heterozygous genotype AG. Also, among the 86 whose ESRD was caused by hypertension, 6.98% expressed the wild-type genotype AA, 13.95% expressed the heterozygous genotype AG and 79.07% expressed the mutant genes.

Table 4. 11: Distribution between Co-morbidity and the genotypic profile

Co-morbidity		**N=87**	**Total Frequency (%)**	***CYP3A4*1B* Frequency (%)**		
		n		AA	AG	**GG**
Diabetes	**Present**	7	8.05	14.29	0.00	85.71
	Absent	80	91.95	6.25	15	78.75
HBP	**Present**	86	98.85	6.98	13.95	79.07
	Absent	1	1.15	0	0	100

N, Total number of genotypes, n; number of patients in a group, HBP; High Blood Pressure, A; Wild-type allele, G; Variant allele. AA= Homozygous wild-type, AG= Heterozygous, GG= Homozygous mutant.

4.6.3 Distribution between etiology and *MDR1_Ex12 (1236 C>T)* genes.

The data shows that the ESRD conditions of the study participants were caused primarily by diabetes and hypertension. Among them, 7 suffered from diabetes and 86 suffered from hypertension. All the 7 diabetic patients expressed the wild-type genotype CC. Also, all the 86 hypertensive patients expressed the wild-type genotype CC.

Table 4. 12: Distribution between Co-morbidity and the genotypic profile

Co-morbidity		**n**	**Total Frequency (%)**	***MDR1_Ex12 (1236 C>T)* Frequency (%)**		
		N=87		CC	CT	**TT**
Diabetes	**Present**	7	8.05	100	0	0
	Absent	80	91.95	100	0	0
HBP	**Present**	86	98.85	100	0	0
	Absent	1	1.15	100	0	0

N, Total number of genotypes, n; number of patients in a group, HBP; High Blood Pressure, C; Wild-type allele, T; Variant allele. CC= Homozygous wild-type, CT= Heterozygous, TT= Homozygous mutant.

4.6.4: Distribution between etiology and *MDR1_Ex21 (2677 G>A)* genes.

The data shows that the ESRD conditions of the study participants were caused primarily by diabetes and hypertension. Among them, 7 suffered from diabetes and 86 suffered from hypertension. All the 7 diabetic patients expressed the wild-type genotype GG. Also, all the 86 hypertensive patients expressed the wild-type genotype GG.

Table 4. 13: Distribution between Co-morbidity and the genotypic profile

Co-morbidity		**n**	**Total Frequency (%)**	***MDR1_Ex21 (2677 G>A)* Frequency (%)**		
		N=87		GG	GA	**AA**
Diabetes	**Present**	7	8.05	100	0	0
	Absent	80	91.95	100	0	0
HBP	**Present**	86	98.85	100	0	0
	Absent	1	1.15	100	0	0

N, Total number of genotypes, n; number of patients in a group, HBP; High Blood Pressure, G; Wild-type allele, A; Variant allele. GG= Homozygous wild-type, GA= Heterozygous, AA= Homozygous mutant.

4.6.5 Distribution between etiology and *MDR1_Ex21 (2677 G>T)* genes.

The data shows that the ESRD conditions of the study participants were caused primarily by diabetes and hypertension. Among them, 7 suffered from diabetes and 86 suffered from hypertension. All the diabetic patients expressed the wild-type genotype GG and none of them expressed the heterozygous genotype GT and mutant genotype TT. Also, among the 86 whose ESRD was caused by hypertension, 98.75% expressed the wild-type genotype GG and the rest (1.25%) expressed the heterozygous genotype GT.

Table 4. 14: Distribution between Co-morbidity and the genotypic profile

Co-morbidity		**n**	**Total Frequency (%)**	***MDR1_Ex21 (2677 G>T)* Frequency (%)**		
		N=87		GG	GT	**TT**
Diabetes	**Present**	7	8.05	100	0	0
	Absent	80	91.95	98.75	1.25	0
HBP	**Present**	86	98.85	85	1	0
	Absent	1	1.15	1	0	0

N, Total number of genotypes, n; number of patients in a group, HBP; High Blood Pressure, G; Wild-type allele, T; Variant allele. GG= Homozygous wild-type, GT= Heterozygous, TT= Homozygous mutant.

4.6.6 Distribution between etiology and *MDR1_Ex26 (3435 C>T)* genes.

The data shows that the ESRD conditions of the study participants were caused primarily by diabetes and hypertension. Among them, 7 suffered from diabetes and 86 suffered from hypertension. Among the patients who had diabetes, 85.71% expressed the wild-type genotype CC and 14.29% expressed the heterozygous genotype CT. None of them expressed the mutant genotype TT. Also, among the 86 whose ESRD was caused by hypertension, 75.58% expressed the wild-type genotype CC, 23.26% expressed the heterozygous genotype CT and 1.16% expressed the mutant genotype TT.

Table 4. 15: Distribution between Co-morbidity and the genotypic profile

Co-morbidity		**n**	**Total Frequency (%)**	***MDR1_Ex26 (3435 C>T)* Frequency (%)**		
		N=87		CC	CT	**TT**
Diabetes	**Present**	7	8.05	85.71	14.29	0
	Absent	80	91.95	73.75	25	1.25
HBP	**Present**	86	98.85	75.58	23.26	1.16
	Absent	1	1.15	0	100	0

N, Total number of genotypes, n; number of patients in a group, HBP; High Blood Pressure, C; Wild-type allele, T; Variant allele. CC= Homozygous wild-type, CT= Heterozygous, TT= Homozygous mutant.

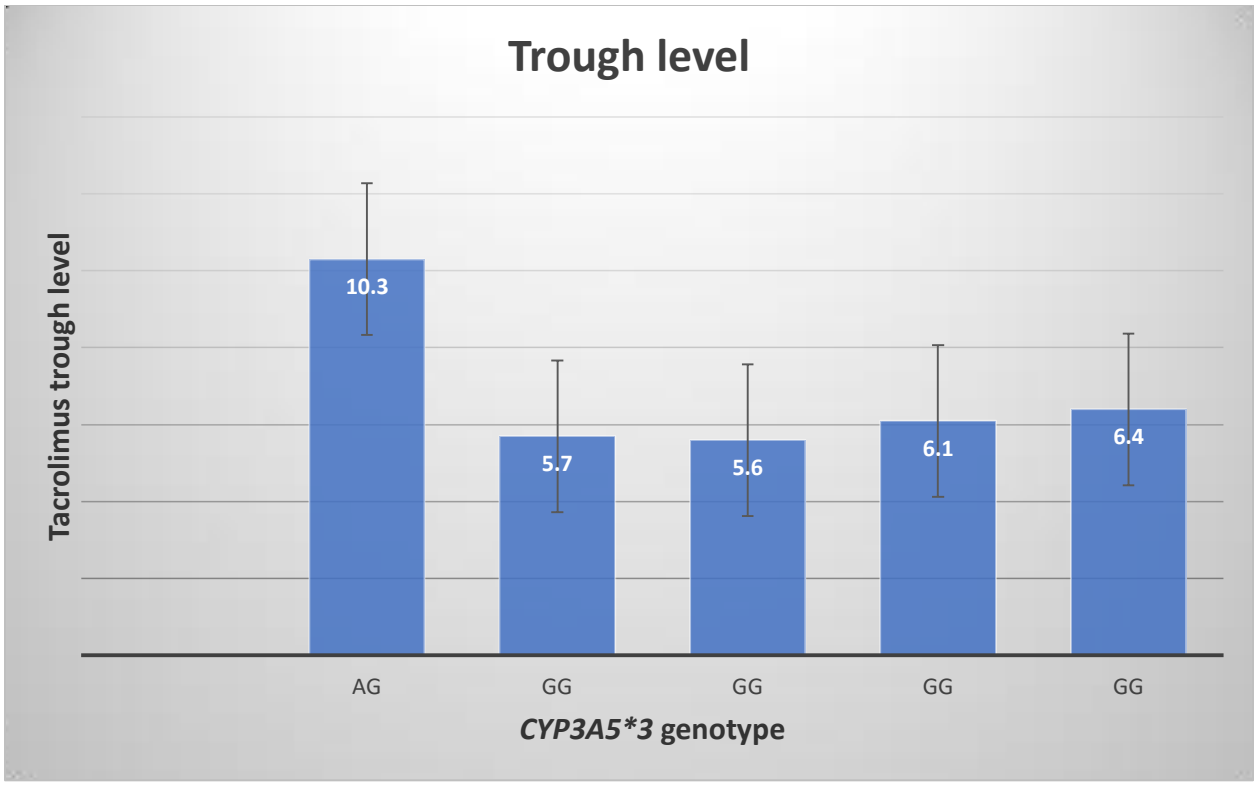

Figure 4. 13: Graph of Tacrolimus trough level against *CYP3A5*3* gene of transplant recipients. The trough level of each recipient is displayed in bar with the concentration indicated on top.

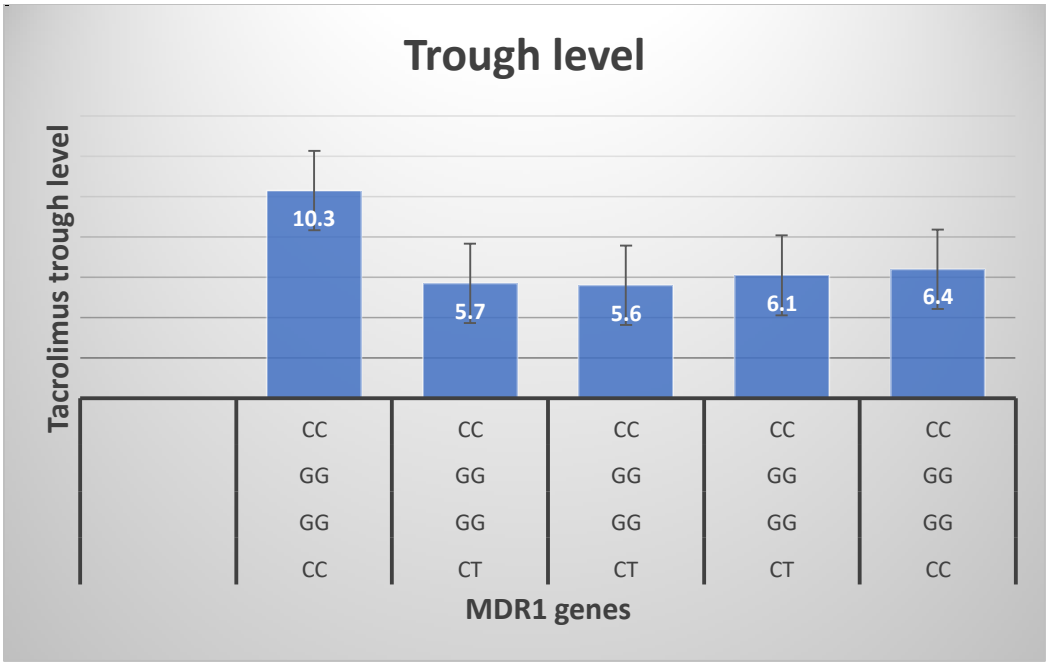

Figure 4. 14: Graph of Tacrolimus trough level plotted against MDR1 haplotype.

MDR1 haplotypes from top to bottom are *MDR1_Ex12, MDR1_Ex21 (G>A), MDR1_Ex21 (G>T) and MDR1_Ex26(C>T)*

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

The Socio-Demographic data obtained from the study participants recorded the mean age as 46 years ±14.39. There were no significant differences between the age groups of ESRD patients. With the exception of the age range 1 - 20, which recorded no patient, the other age ranges had significantly high frequencies (Table 4.1). This supports the findings that all age groups have an equal exposure to ESRD, since the disease conditions that pre-disposes one to ESRD affect all age groups (Gebeyehu et al., 2010). All the patients suffered from at least one disease condition that leads to the deterioration of their kidney (Table 4.2). The etiology of 86 (98.85%) of the ESRD patients was hypertension. Diabetes was recorded as the second leading cause of ESRD. The data collected showed that none of the patients had the etiology of the ESRD from Nephritic Syndrome or Chronic Glomerulonephritis, although the progression of the ESRD may have caused Chronic Glomerulonephritis.

Out of the 82 patients receiving dialysis therapy, 69 (84.15%) of them were under medications to manage their ESRD condition and other comorbidities (Table 4.3). In a study, if the blood pressure of patients remains normal in between dialysis period and does not exceed 160/95 mmHg prior to the next dialysis session, the antihypertensive therapy is withheld (Stern et al., 2014). Among the list of medications that are administered to dialysis patients, Amlodipine and Methyldopa top the chart with 24 (32.93%) and 25 (30.49%) respectively. This is followed by other antihypertensive like Bisoprolol 18 (21.95%). Although the study shows no significance difference between patients taking analgesics and those who do not take analgesics, 89.13% of

those taking analgesics take Paracetamol. Studies done shows that CYP3A4 contributes to 20% total metabolism of Paracetamol, especially at lower doses (Kalsi et al., 2011; Laine et al., 2009). Hence SNPs in CYP3A4 will affect the rate their enzymes metabolizes Paracetamol and other medication administered that are substrate of CYP3A4 enzyme.

The genetic frequencies of *CYP3A5*3 (6986 A>G)* recorded in Table 4.4 shows that among the 87 study participants, a significant number of 63 (72.40%) patients expressed the wild-type *CYP3A5*1/*1*, 20 (23%) expressed the heterozygous *CYP3A5*1/*3* and 4 (4.60%) expressed the homozygous mutant *CYP3A5*3/*3*. This findings is similar to findings in a study done in the Ghanaian population that showed that 15% of the populace express the variant allele *CYP3A5*3* (Kudzi et al., 2010). This finding indicates that a significant number of the patients are normal metabolizers of Tacrolimus and other medications received by dialysis patients (anti-hypertensive and anti-diabetic) that are substrate to CYP3A5. That notwithstanding, a significantly high variant allele (G) frequency of 28% was observed to be expressed in this study. This findings show that there are still significant number of patients poorly metabolizing Tacrolimus and other CYP3A5 substrate medications like the anti-hypertensive and anti-diabetic that dialysis patients receive daily. Individuals expressing the mutant CYP3A5 enzymes are at risk of experiencing the adverse effects associated with Tacrolimus overdose. This is due to high concentrations of such medications in the blood; especially with it narrow therapeutic index. This becomes very worrying as most of these dialysis patients have been on medications that are substrate to CYP3A5 gene for the length of years. The trough levels of these medications need to be monitored in ESRD patients and their doses varied as well. This is because a large number of such medications are daily administered to patients. Among the transplant recipients, no such mutant genotype *CYP3A5*3* (G) was expressed, implying that these patients express the normal

levels of the CYP3A5 enzymes in their bodies. Also from Hardy-Weinberg equilibrium analysis for CYP3A5, a value of 0.9858 was obtained. This is less than 1 and as such it could be said that the population is evolving as there are changes in allele frequencies. This could be due to natural selection or existence of mutations of some genotypes for better survival of organisms.

The genetic frequencies of *CYP3A4*1B (-290 A>G)* recorded in Table 4.5 shows that among the 87study participants, 6 (6.90%) of them expressed the wild-type genotype AA, 12 (13.79%) expressed the heterozygous genotype AG and 69 (79.31%) expressed the mutant genotype GG. The data shows that a significant number of ESRD patients express the mutant genotype. Mutation in the *CYP3A4* gene is linked to enhanced activity of the enzyme and or an up-regulation of the enzyme, causing rapid metabolism of medications including Tacrolimus (Shi et al., 2015). Homozygous mutant genotype (GG) metabolizes medications faster than heterozygous carrier genotype (AG). This shows that medications that are substrate of this enzyme will be metabolized faster in the homozygous mutant *CYP3A4*1B/*1B*, than in the carrier *CYP3A4*1/*1B* and the wild-type *CYP3A4*1/*1*. Patients expressing the mutant allele G will experience a lower drug trough level and the therapeutic effect will not be achieved. The study also showed that 4 (80%) of transplant recipient expressed the mutant genotype GG and as such those patients are observed to have low Tacrolimus trough concentration. This follows the findings that such patients will experience low Tacrolimus trough level as the drug is metabolized faster (Roy et al., 2006). Also from Hardy-Weinberg equilibrium analysis for CYP3A4, a value of 0.91 was obtained. This is less than 1 and as such it could be said that the population is evolving as there are changes in allele frequencies. This could be due to natural selection or existence of mutations of some genotypes for better survival of organisms.

The frequency of *MDR1_Ex12 (1236 C>T)* gene recorded showed that all the participants expressed the wild-type gene (Table 4.6). A similar genotypic frequency of 100% was seen in the Ghanaian populace from a study (Kudzi et al., 2010). SNPs in this gene is silent and does not produce a different amino acid (Ieiri et al., 2004). The presence of C allele in all the participants shows that normal levels of these genes are expressed in them. As such *MDR1_Ex12 (1236 C>T)* gene does not affect the rate at which administered drugs reach systemic circulation and target points. Also from Hardy-Weinberg equilibrium analysis for *MDR1_Ex12 (1236 C>T)*, a value of 1 was obtained, and this shows that there are no changes in allele frequencies as none of the gene is any better than the other for the organisms survival.

The frequency of *MDR1_Ex21 (2677 G>A)* gene obtained showed that all participants had the wild-type genotype GG (Table 4.7). This findings is similar to a study where a gene frequency of 100% found in the Ghanaian population (Kudzi et al., 2010). None of the participants carried the MDR1 variant allele A. This finding shows that the normal genes are expressed and the concentration of medications reaching their site of action and the systemic circulation are not affected by the proteins expressed by the *MDR1_Ex21 (2677 G>A)* gene. Also from Hardy-Weinberg equilibrium analysis for *MDR1_Ex21 (2677 G>A)*, a value of 1 was obtained, and this shows that there are no changes in allele frequencies as none of the gene is any better than the other for the organisms survival.

The data obtained from *MDR1_Ex21 (2677 G>T)* genotyping showed that 86 (98.85%) of the study participants expressed the wild-type genotype GG, 1 (1.15%) expressed the heterozygous genotype GT and none of them expressed the mutant genotype TT (Table 4.8). A variant allele frequency of 0.57% from the participants showed that some of the subjects have a reduced expression of intestinal P-gp and such participants would have a higher plasma concentration of

a drug than individuals expressing only the wild-type allele. This would be supported from a study that shows that the absolute bioavailability from both oral and intravenous administrations of digoxin was significantly higher in expressers of variant allele T (Ieiri et al., 2004). None of the transplant recipients expressed the variant allele. Also from Hardy-Weinberg equilibrium analysis for *MDR1_Ex21 (2677 G>T)*, a value of 1 was obtained, and this shows that there are no changes in allele frequencies as none of the gene is any better than the other for the organisms survival.

The data obtained from *MDR1_Ex26 (3435 C>T)* genotyping showed that 65 (74.71%) of the study participants expressed the wild-type genotype CC, 21 (24.14%) expressed the heterozygous genotype CT and 1 (1.15%) expressed the mutant genotype TT (Table 4.9). Although a very small percentage of the patients expressed the variant genotype TT, a significant number of variant allele was expressed from the total participants. Expression of the variant allele reduces the expression of the protein in the duodenum, thus causing an increase plasma concentration of Tacrolimus and other drugs that are substrate of *MDR1_Ex26 (3435 C>T)*. This inference would be supported from a study that subject carrying homozygous variant of this gene showed a significantly reduced duodenal expression of this protein (Wolking et al., 2015). Among the transplant recipients, none expressed the mutant genotype TT but 3 (30%) out of the 10 allele expressed were the mutant allele T. Also from Hardy-Weinberg equilibrium analysis for *MDR1_Ex26 (3435 C>T)*, a value of 1.04 was obtained. This is greater than 1 and as such it could be said that the population is evolving as there are changes in allele frequencies. This could be due to natural selection or existence of mutations of some genotypes for better survival of organisms.

Data from CYP450 genotyping showed that different genotypes were expressed at varying frequencies in relation to the etiology of ESRD. The data obtained from *CYP3A5*3 (6986 A>G)* genotyping showed that among the 7 patients diagnosed with diabetes, 57.14% expressed the wild-type *CYP3A5*1/*1* and 43.86% expressed the heterozygous *CYP3A5*1/*3*; also among the 86 patients diagnosed with hypertension,72.09% expressed the wild-type *CYP3A5*1/*1*, 23.26% expressed the heterozygous CYP3A5*1/*3 and 4.65% expressed the variant CYP3A5*3/*3 (Table 4.10). A study done showed that the hypertension or diabetes disease condition does not affect the expression levels of CYP3A5 mRNA or protein in humans (Yamada et al., 2007). Also the data obtained from *CYP3A4*1B (-290 A>G)* genotyping showed that among the 7 ESRD patients whose disease etiology was diabetes, 14.29% expressed the wild-type *CYP3A4*1/*1* and 85.71% expressed the variant *CPY3A4*1B/*1B*; also among the 86 ESRD patients whose disease etiology were both diabetes and hypertension, 6.98% expressed the wild-type *CYP3A4*1B/*1B*, 13.95% expressed the heterozygous *CYP3A4*1/*1B* and 79.07% expressed the *CYP3A4*3/*3* (Table 4.11). This result indicates that large number of diabetics and hypertensive patients expressed the variant gene allele. In a study done, it was observed that diabetes significantly decreases protein level and enzymatic activity of CYP3A4 (Coto et al., 2010).

Data obtained from MDR1 genotyping didn't show any pattern in the genotypic frequencies and the etiology of ESRD. Both diabetic and hypertensive patients expressed the wild-type genotype CC and GG for *MDR1_Ex12 (1236C>T)* and *MDR1_Ex21 (2677G>A)* respectively (Table 4.12 and Table 4.13). A similar result was obtained from patients in a study where there was on observed association between MDR1 polymorphism and diabetes or hypertension disease condition (Ergen, 2013).

The result of *CYP3A4*1B* genotype showed that, one out of the five patients who had received transplants expressed the heterozygous *CYP3A4*1/*1B* gene whilst the other four transplant recipients expressed the variant gene *CYP3A4*1B/*1B*. From figure 4.13 it was seen that the trough level of the transplant recipient with heterozygous *CYP3A4*1B/*1B* was higher compared to the trough levels of those who expressed the homozygous *CYP3A4*1B/*1B* variant. This is in line with the findings that mutation in *CYP3A4*1B* gene causes an enhanced enzyme activity or over expression of the CYP3A4 enzyme resulting in a reduced drug concentration level (Coto et al., 2010; Shi et al., 2015). This result shows that transplant patients expressing the variant gene metabolize Tacrolimus faster. Among the MDR1 haplotypes (figure 4.14), only *MDR1_Ex26* genotype recorded the expression of the variant gene. The substitution of C with T in *MDR1_Ex26 (C>T)* is associated with increased Tacrolimus trough level (Ieiri et al., 2004). This is because this mutation leads to reduced expression of P-glycoprotein in the duodenal wells. This finding shown in figure 4.14 was in contrast to the findings observed in a study by Ieiri et al in 2004.

5.2 Conclusion

This study established an interesting pattern in the Tacrolimus trough level and *CYP3A4*1B* genes expressed among transplant recipients, although the number of transplant recipients recruited into the study was small. Those expressing the homozygous variant gene *CYP3A4*1B/*1B* had a lower Tacrolimus trough level than those who expressed the heterozygous gene *CYP3A4*1/*1B*.

The allele and genotypic frequencies of *CYP3A5*3* and *CYP3A4*1B* genes showed that a sizeable number of variant allele G and G respectively were expressed among the patients. These expressed variants play a key role in the variation of the pharmacokinetics of Tacrolimus, anti-hypertensive, anti-diabetics and others.

Varying expression levels of MDR1 gene that expressed the P-gp transporter proteins were observed within the study participants. From the genotyping results of MDR1, participants expressed only the wild-type gene for *MDR1_Ex12 (1236C>T)* and *MDR1_Ex21 (2677G>A)* but a number of mutant alleles were expressed in the *MDR1_Ex21 (2677G>T)* and *MDR1_Ex26 (3435 C>T)* genes.

RECOMMENDATION

It is recommended that a large number of study participants especially the transplant recipients be recruited into the study so that a strong correlation can be established among transplant recipients and Tacrolimus trough levels within the Ghanaian population.

LIMITATIONS

Most of the ESRD patients did not want to be recruited into this study because they had not received feedback from previous researches they had been involved.

Also transplant patients were not easily accessible as most of them do not attend regular review at the hospital but instead prefer to either travel outside the country or meet their health providers at their private clinics for review.

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APPENDIX I: Reagents and Equipment

Reagents:

1. Nuclease free water
2. Ethidium bromide
3. One Taq Quick-Load 2X Master Mix with Standard Buffer
4. 50X TAE Buffer
5. Distilled Water
6. Quick-Amp DNA extraction kit
7. Quick-Load Purple 100bp DNA ladder
8. Double distilled water
9. NaOH
10. Absolute and 70% ethanol
11. Agarose powder

Equipment:

1. GeneAmp PCR System 9700 (Certified Scientific Instruments, Inc)
2. Autoclave
3. Microwave Oven
4. Micro Pipettes (0.5-20uL, 5-50uL, 20-200uL, 100-1000uL)
5. Agarose Gel Electrophoresis Systems (Bio-Rad Laboratories, Inc)
6. Gel Doc EZ Imager System (Bio-Rad Laboratories, Inc)
7. NanoDrop Lite Spectrophotometer (Thermo Scientific, Inc)

8. Printer
9. Measuring cylinder
10. Glass stirrer
11. pH Meter
12. Huma Twist Vortex (Human GmbH, Max-Planck-Ring 21 65205)
13. Water Bath (Memmert GmbH Co.)
14. PCR tubes (0.2ml)
15. Eppendorf tubes (2.5ml)
16. Spatula
17. Centrifuge 5418R (Eppendorf AG, USA)
18. Analytical balance (Ohaus Corporation, USA)

APPENDIX II: Preparation of reagents

Preparation of 1X TAE Buffer

A volume of 10ml stock 50X TAE buffer was measured into a 500ml volumetric flask. The volume was adjusted to the 500ml mark with double distilled water. The flask was covered and inverted several times for thorough mixing. The prepared working buffer was stored at room temperature for use.

Agarose gel (2% w/v) preparation

Agarose powder (1.5g) was weighed using an analytical electronic balance into a heat-resistant glass bottle and 75ml of 1X TAE buffer was added to it. The mixture was swirled severally and allowed to melt in a microwave oven for about 60 seconds. The solution was allowed to cool just above room temperature. Ethidium bromide (3µL) was added and mixed gently by swirling. The solution poured into the casting tray and the combs were inserted to create wells in the gel. Agarose gel (2%) was prepared for running PCR solution after PCR amplification.

Agarose gel (3%) was prepared to run solutions after restriction digestion. For 3% agarose gel, 2.25g of agarose powder was weighed and dissolved into 75ml of 1X TAE buffer.

Preparation of 100µM Primer Stock Solution

These calculations were done based on the equation:

Concentration (C) = Amount of substance (n) / Volume (V)

1. ***CYP3A5*3 (6986 A>G)***

A. *CYP3A5*3 (6986 A>G)* Forward primer

Mole (n) = 23.38nmol

Concentration (C) = 100µM

Volume (V) = n/c = $(23.38\times10^{-9}\text{mols})/ (100\times10^{-6}\text{mol/dm}^3)$

V = 0.2338×10^{-3}L = 233.8µL

Therefore, 100µM of the stock solution is prepared by adding 233.8µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (23.38nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

B. *CYP3A5*3 (6986 A>G)* Reverse primer

Mole (n) = 26.85nmol

Concentration (C) = 100µM

Volume (V) = n/c = $(26.85\times10^{-9}\text{mols})/ (100\times10^{-6}\text{mol/dm}^3)$

V = 0.2685×10^{-3}L = 268.4µL

Therefore, 100 µM of the stock solution is prepared by adding 268.5 µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (26.85nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

2. ***CYP3A4*1B (-290 A>G)***

A. *CYP3A4*1B (-290 A>G)* Forward primer

Mole (n) = 62.7 nmol

Concentration (C) = 100 µM

Volume (V) = n/c = (62.70×10^{-9} mols)/ (100×10^{-6} mol/dm^{3})

V = 0.627×10^{-3} L = 627 µL

Therefore, 100 µM of the stock solution is prepared by adding 627 µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (62.7 nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

B. *CYP3A4*1B (-290 A>G)* Reverse primer

Mole (n) = 55.32 nmol

Concentration (C) = 100 µM

Volume (V) = n/c = (55.32×10^{-9} mols)/ (100×10^{-6} mol/dm^{3})

V = 0.5532×10^{-3} L = 553.2 µL

Therefore, 100 µM of the stock solution is prepared by adding 553.2 µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (55.32 nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

3. *MDR1_Ex12 (1236 C>T)*

A. *MDR1_Ex12 (1236 C>T)* Forward primer

Mole (n) = 37.61 nmol

Concentration (C) = 100 µM

Volume (V) = n/c = $(37.61\times10^{-9}$ mols)/ $(100\times10^{-6}$ mol/dm$^3)$

V = 0.3761×10^{-3} L = 376.1 µL

Therefore, 100 µM of the stock solution is prepared by adding 376.1 µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (37.61 nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

B. *MDR1_Ex12 (1236 C>T)* Reverse primer

Mole (n) = 44.95 nmol

Concentration (C) = 100 µM

Volume (V) = n/c = $(44.95\times10^{-9}$ mols)/ $(100\times10^{-6}$ mol/dm$^3)$

V = 0.4495×10^{-3} L = 449.5 µL

Therefore, 100 µM of the stock solution is prepared by adding 449.5 µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (44.95 nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

4. *MDR1_Ex26 (3435 C>T)*

A. *MDR1_Ex26 (3435 C>T)* Forward primer

Mole (n) = 60.92nmol

Concentration (C) = 100µM

Volume (V) = n/c = (60.92×10^{-9}mols)/ (100×10^{-6}mol/dm^3)

V = 0.6092×10^{-3}L = 609.2µL

Therefore, 100µM of the stock solution is prepared by adding 609.2µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (60.92nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

B. *MDR1_Ex26 (3435 C>T)* Reverse primer

Mole (n) = 76.03nmol

Concentration (C) = 100µM

Volume (V) = n/c = (76.03×10^{-9}mols)/ (100×10^{-6}mol/dm^3)

V = 0.7603×10^{-3}L = 760.3µL

Therefore, 100µM of the stock solution is prepared by adding 760.3µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (76.03nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

5. *MDR1_Ex21 (2677 G>T)*

A. *MDR1_Ex21 (2677 G>T)* Forward primer

Mole (n) = 62.59nmol

Concentration (C) = 100µM

Volume (V) = n/c = (62.59×10^{-9}mols)/ (100×10^{-6}mol/dm^3)

V = 0.6259×10^{-3}L = 625.9µL

Therefore, 100µM of the stock solution is prepared by adding 625.9µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (62.59nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

B. *MDR1_Ex21 (2677 G>T)* Reverse primer

Mole (n) = 68.17nmol

Concentration (C) = 100µM

Volume (V) = n/c = (68.17×10^{-9}mols)/ (100×10^{-6}mol/dm^3)

V = 0.6817×10^{-3}L = 681.7µL

Therefore, 100µM of the stock solution is prepared by adding 681.7µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (68.17nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

6. *MDR1_Ex21 (2677 G>A)*

A. *MDR1_Ex21 (2677 G>T)* Forward primer

Mole (n) = 62.59nmol

Concentration (C) = 100µM

Volume (V) = n/c = (62.59×10^{-9}mols)/ (100×10^{-6}mol/dm^3)

V = 0.6259×10^{-3}L = 625.9µL

Therefore, 100µM of the stock solution is prepared by adding 625.9µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (62.59nmol). It was then vortex for 10 seconds and stored at -20^{O}C.

B. *MDR1_Ex21 (2677 G>T)* Reverse primer

Mole (n) = 76.03nmol

Concentration (C) = 100µM

Volume (V) = n/c = (76.03×10-9mols)/ (100×10-6mol/dm3)

V = 0.7603×10-3L = 760.3µL

Therefore, 100µM of the stock solution is prepared by adding 760.3µL of sterile double distilled water (pH 7.6) to the lyophilized primer supplied (76.03nmol). It was then vortex for 10 seconds and stored at -20OC.

APPENDIX III: Primer sequences, amplicon sizes and Restriction Enzymes

Genes	Primer sequence 5'-3'	Restriction enzyme	PCR Amplicon size (bp)
*CYP3A4*1B (-290 A>G)*	FP: GGACAGCCATAGAGACAACTGCA RP: CTTTCCTGCCCTGCACAG	Pst I	334
*CYP3A5*3 (6986 A>G)*	FP: CATCAGTTAGTAGACAGATGA RP: GGTCCAAACAGGGAAGAAATA	Ssp I	293
MDR1_Ex12 (1236 C>T)	FP: TATCCTGTGTCTGTAATTGCC RP: CCTGACTCACCACACCAATG	Hae III	366
MDR1_Ex26 (3435 C>T)	FP: TGCTGGTCCTGAAGTTGATCTGTGAAC RP: ACATTAGGCAGTGACTCGATGAAGGCA	Mbo I	248
MDR1_Ex21 (2677 G>A)	FP: TGCAGGCTATAGGTTCCAGG RP: GTTTGACTCACCTTCCCAG	Bsr I	220
MDR1_Ex21 (2677 G>T)	FP: TGCAGGCTATAGGTTCCAGG RP: TTTAGTTTGACTCACCTTCCCG	Ban I	224

FP= Forward primer, RP= Reverse primer

APPENDIX IV: Consent Form

GENETIC POLYMORPHISMS AFFECTING TACROLIMUS DOSE REQUIREMENTS IN GHANAIAN PATIENTS WITH END-STAGE RENAL DISEASE

Tacrolimus is an important drug used in the management of patients after kidney transplant. The effects of incorrect doses of Tacrolimus range from the body rejecting the newly transplanted kidneys to adverse drug reaction which can even result in death. It is therefore important that patients who have had kidney transplant and are being managed on Tacrolimus, are given doses that will help the body not to reject it and also have very minimal adverse reaction. There is evidence that enzymes that are responsible for the breakdown of Tacrolimus in the body can vary due to the genes. This can lead to differences blood concentrations of Tacrolimus per dose in different people. The results from this study will be useful to clinicians managing dose/response better among Ghanaian Kidney transplant patients on Tacrolimus. The study will also contribute knowledge to the pharmacogenomic database for Ghana.

This study will take 3ml of blood from you by inserting a needle in your forearm. The risk involved in this blood collection procedure is negligible and it will cause only minimal pain and bruising.

It will be appreciated if you will agree to take part in this study. Your participation in the study is voluntary, and you can leave the study at any time without any disadvantage concerning your medical care at this Clinic. All information gathered will be treated in strict confidentiality.

If you have further questions on the study you can contact:

Dr Vincent Boima Telephone number:

Dr Edward PK Kwakyi Telephone number: 0202536092

Email Address: edwardkwakyi@gmail.com

Consent:

I …… (PRINT)

of…….

Give my consent for my sample to be used for the research project stated above which has been explained to me

By……

Patient signature…….....Date……

- In case of language interpretation; witness

Witnesses' Name……Date……

Signature…….

Doctor's signature……Date…….

APPENDIX V: Data Collection Sheet

Genetic polymorphisms affecting Tacrolimus dose requirements in Ghanaian patients with End –Stage Renal Disease

Date: …. /……/…… Patient ID: ……. Study ID: ……

Section A:

1. **Please record the socio-demographic data of the subject**

Age: …… Sex: [ ] male [ ] Female

Ethnicity: …….

Region of permanent residence: ……

Marital status: [ ] Single [ ] Married [ ] Divorced [ ] Widowed

[ ] Co-habitation [ ] LGBT

Occupation: …….

2. **Clinical History**

a. Diabetes [ ] Duration: ……..

b. Hypertension [ ] Duration: ……..

c. Sickle cell disease [ ] Duration:

Genotype: ……

d. Systemic Lupus Erythematosus [ ] Duration: ……..

e. Nephrotic Syndrome [ ] Duration: ……..

f. Chronic Glomerulonephritis [ ] Duration: ……..

g. Have you had a Kidney biopsy Yes [ ] No [ ]

h. If yes, what was the histological diagnosis……

3. Other risk factors

a. Have you previously been managed for Acute Kidney Injury (AKI): Yes [ ] No [ ]

b. If yes, how long ago? …….

c. Do you regularly use "*pain killers"*? Yes [ ] No [ ]

If yes, what's the name …….

How often? Daily [ ] Weekly [ ] Every 2 weeks [ ]

Monthly [ ] Occasional [ ]

d. Do you use any herbal preparations? Yes [ ] No [ ]

If yes, what's the name …….

If yes, how often? Daily [ ] Weekly [ ] Every 2 weeks [ ] Monthly [ ]

e. Do you have any first-degree relatives who have suffered from a kidney disease?

Yes [ ] No [ ]

4. Current medications (Please tick where appropriate

ANTIHYPERTENSIVES

a. Nifedipine
b. Amlodipine
c. Felodipine
d. Ramipril
e. Lisinopril
f. Captopril
g. Losartan
h. Valsartan
i. Candesartan
j. Atenolol
k. Carvedilol
l. Bisoprolol
m. Metoprolol

DIURETIC

a. Furosemide
b. Spirinolactone

IMMUNOSUPPRESANTS

a. Prednisolone
b. Azathioprine
c. Azathioprine
e. Methotrexate
d. Hydroxychloroquine
f. Mycophenolate mofetil

MISCELLANEOUS

a. Digoxin
d. Calcium carbonate
b. Erythropoietin
e. Alfa calcidol
c. Iron replacement
f. Calcium Resonium

Others (specify)…….....

NON-TRANSPLANT PATIENTS

a. Are you currently on hemodialysis? Yes [ ] No [ ]
b. If yes, how often do you receive hemodialysis?

   Once a week [ ], Twice a week [ ], 3 times a week [ ], other (specify)……
c. What vascular access are you currently using for hemodialysis?

Temporary neck line [ ], Perm Cath [ ], Femoral line [ ], Fistula [ ], Graft [ ]

[ ] Other (specify)……..

d. How many times have you been hospitalized in the last 6 months?

Once [ ], 1-5 times [ ], 5-10 times [ ], >10 times [ ]

e. What is your average duration of admission?

< 1 week [ ], 1-2 weeks [ ], > 2weeks [ ]

f. Do you attend follow up clinics regularly and on time? Yes [ ] No [ ]

TRANPLANT RECIPIENTS

BASIC INFORMATION

a. How long ago did you have your transplant? < 6 months [ ], < 1 year [ ]

1- 5 years [ ], > 5 years [ ]

b. What type of transplant did you receive? Living donor [ ] cadaveric [ ]

c. How are you related to your donor? Parent [ ] Child [ ] Sibling [ ]

Cousin [ ] Unrelated [ ]

d. Where was your transplant surgery done? Ghana [ ] South Africa [ ]

USA [ ] UK [ ], Europe [ ] India [ ] Other (specify)……

e. Do you attend follow up clinics regularly and on time? Yes [ ] No [ ]

COMPLICATIONS

a. Have you been hospitalized in the last 6 months? Yes [ ] No [ ]

b. If yes, how many times? Once [ ] 1-5 times [ ] 5-10 times [ ],

> 10 times [ ]

c. What is your average duration of admission? 1 week [ ] 1-2 weeks [ ]

> 2weeks [ ]

d. Have you developed any complications as a result of your transplant?

Yes [ ] No [ ]

If yes, please specify in box below (as many as apply to you)

Post-operative wound infection		Delayed wound healing/infection	
Hyperacute rejection		Tuberculosis	
Acute rejection		CMV complications	
Chronic rejection		Drug side effects	
Post-transplant diabetes		Recurrent urinary tract infections	
Obstructive uropathy		Depression	
Kidney stones		Others (specify)	

TACROLIMUS

a. What was your initial dose of Tacrolimus? ……..

b. What is your current dose of Tacrolimus? ……..

c. Have you experienced any adverse effects that you attribute to Tacrolimus?

Yes [ ] No [ ]

If yes, please specify in box below (as many as apply to you)

Tremors	
Hypertension	
Diabetes mellitus	
Renal impairment	
Infections	
Headache	
Leucopenia	

Others (specify)…….....

d. Were these confirmed as accurate by your doctor? Yes [ ] No [ ]

e. How compliant are you with regards to Tacrolimus?

Always [ ] Very often [ ] Often [ ] Poor [ ]

LABORATORY RESULTS

FULL BLOOD COUNT

HB	
MCH	
MCV	
WBC	
PLATELETS	

BUE and Creatinine

Sodium (mmol/l)	
Potassium (mmol/l)	
Chloride (mmol/l)	
Bicarbonate (mmol/l)	
Urea (mmol/l)	
Creatinine (umol/l)	

BIO DATA

WEIGHT (Kg)	
HEIGHT (M)	
BODY MASS INDEX (Kg/m^2)	
BODY SURFACE AREA (m^2)	