Development and Validation of a Pharmacogenomics Profiling Panel Suitable for Personalizing Metformin Therapy

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ABSTRACT

Development and Validation of a Pharmacogenomics Profiling Panel Suitable for Personalizing Metformin Therapy

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The burden of non-communicable diseases (NCDs) in South Africa is predicted to increase substantially in the next decades if the necessary preventative measures are not taken. The two most common NCDs associated with rapid mortality increase are diabetes mellitus (DM) and hypertension (HTN). Both of these diseases, i.e DM and HTN, can be a result of a combination of modifiable risk factors (behavioral) and non-modifiable risk factors (genetic, physiological, and environmental). New strategies implemented to manage these diseases should include addressing both modifiable and non-modifiable risk factors for patients with NCDs. The aim of this study was to contribute to the reduction of incidence of uncontrolled T2DM among patients taking metformin as a first-line anti-diabetic drug, through the development of individualized therapy for this drug. When implemented, this could be one of the healthcare strategies to address non-modifiable risk factors for patients with T2DM as an important NCD. The first objective of the study was to explore the prevalence and risk factors of DM and HTN in South Africa, especially within the economically disadvantaged population. A cross-sectional analytical study was conducted in the Cecilia Makiwane Hospital serving the residents of Mdantsane from July 2017 – October 2017. Socio-demographic data, anthropometric measurements, triplicate blood pressure, fasting blood glucose and lipogram

analysis were obtained from 265 outpatients (18 years and older). Multivariate analysis showed “no salt intake”, “never smoke”, “normal” triglyceride and decreased high-density lipoprotein levels were significantly associated with a reduction of DM with adjusted odds ratio of 0.12 (95% CI:0.03-0.43; *p*=0.001), 1.23 (95% CI: 0.73-2.06; *p*=0.013) and 0.16 (95% CI: 0.21-1.29; *p*=0.003), respectively. Underweight and normal-weight were significantly associated with a decreased risk of hypertension with odds ratio of 7.98 (95% CI: 2.02- 31.53; *p*=0.003) and 19.17 (95% CI: 2.53-145.20; *p*=0.004), respectively. More importantly, this investigation highlighted the extent of uncontrolled DM and HTN among resource-constrained patients receiving treatment in Cecilia Makiwane hospital, serving the rural areas in Mdantsane. The second objective of the study was to evaluate the suitability of nineteen pharmacogenomics biomarkers for individualized metformin therapy for T2DM patients. A genetic association study was conducted to investigate the level of association between nineteen pharmacogenomics biomarkers (SNPs) and response to metformin treatment, and to evaluate their suitability for individualizing metformin therapy for diabetic patients from the Bantu populations. Two multiplex MassARRAY systems (Agena BioscienceTM) were designed and optimized by Inqaba Biotechnical Industries (Pretoria, South Africa), and used for the genotyping of the selected SNPs for 140 T2DM outpatients. The CT genotype of the *FMO2* rs12752688 polymorphism was significantly associated with increased response to metformin therapy (OR= 0.33, 95% CI [0.16-0.68], *p*-value= 0.003). A moderate association was also found between the GA genotype of *SLC47A2* rs12943590 and a decreased response to metformin therapy (OR= 2.29, 95% CI [1.01-5.21], *p*- value=0.048 for the

heterozygous GA genotype. The *FMO2* rs12752688 polymorphism is suggested to be included in pharmacogenomics profiling systems developed to individualize metformin therapy for diabetic patients from the Bantu populations.

DECLARATION

I declare that 'Development and Validation of a Pharmacogenomics Profiling Panel Suitable for Personalizing Metformin Therapy" is my own work that has not been submitted for any degree or examination in any other university and that all the sources I have used have been indicated and acknowledged by complete references.

Full Name: Lettilia Xhakaza **Date: March 2019**

Signed: 

Dedicated to my mom and dad

Nombango Florence and Bhozana Request Milton Sydwell Xhakaza

Thank you for your endless love

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LIST OF ABBREVIATION

ABCC8	ATP-binding cassette transporter sub-family C member 8
ADA	American Diabetes Association
ADME	Absorption, distribution, metabolism and elimination
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATM	Ataxia telangiectasia mutated
ATP	adenosine phosphate
BMI	Body mass index
BP	Blood pressure
CVD	Cardiovascular disease
CYP450	Cytochrome 450
DDI	Drug-drug interaction
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
EXO I	Exonuclease I
FBG	Fasting blood glucose
FDA	Food and Drug Administration
FMO	Flavin monooxygenases
GLUT4	Glucose transport type 4
HBA1C	Glycosylated Hemoglobin
HDL	High density lipoprotein
HTN	Hypertension
HWE	Hardy-Weinberg equilibrium

IDF	International Diabetes Federation
INR	International nomalised ratio
KATP	ATP k+ channel
KCJII	potassium inwardly-rectifying channel, subfamily J, member II
KIF6	Kinesin-like family 6
Kir6.2	potassium inward rectifier 6.2
LDL	Low density lipoprotein
LKB1	Liver kinase B1
MALDI-TOF-MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MATE	multidrug and toxins extrusion protein
MPP	I-methyl-4-phenylpyridinium
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NCD	Non-communicable disease
OAD	Oral anti-diabetic drug
OCT	Organic Cation Transporter
PBG	Postprandial blood glucose
PCR	Polymerase chain reaction
PharmGKB	Pharmacogenomics knowledge base
PMAT	Plasma membrane monoamine transporter
PPG	Postprandial plasma glucose
RCT	Randomized clinical trial
SAP	Shrimp Alkaline Phosphatase

SLCO1B1	solute carrier organic anion transporter family member 1B1
SLC	Solute Carrier Transporter
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SSR	Microsatellites
STK11	Serine/Threonine Kinase 11
SU	Sulfonylureas
SURI	sulfonylurea receptor I
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TEA	Tetraethylammonium
TG	Triglyceride
TPMT	Thiopurine methyltransferase
TZD	Thiazolidinedione
VKORC1	vitamin K epoxide reductase complex subunit 1
WHO	World Health Organization

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Chapter One

Literature review

1.1. Introduction

South Africa is one of the countries experiencing an increasing burden of non-communicable diseases (NCDs). NCDs are the major source of mortality and morbidity, which is estimated to surpass the burden of infectious diseases by 2035. Past decades studies have been focusing on infectious diseases and much less research has been conducted on NCDs (WHO, 2018a; Dalal *et al*, 2011; Omoleke, 2013 Levitt *et al*, 2011). The world health organization (WHO) estimates that seven out of ten deaths are to occur due to these diseases by the year 2020 with the prevalence of the four related clusters diseases i.e. cardiovascular diseases (CVD), cancer, diabetes mellitus (DM) and chronic respiratory diseases. Approximately three-quarter of all NCDs deaths occur in low- and middle- income countries (including South Africa) (WHO, 2018a; Lozano *et al*, 2012; Lim *et al*, 2012). Moreover, in South Africa, NCDs occurs in both rural and urban areas however, most studies are done in urban areas (Mayosi *et al*, 2009). In 2016, it had 265000 (51%) of NCDs death from countries deaths (WHO, 2016). Therefore, since these diseases are recognized as a public health problem, countries need to implement strategies to better the economic growth and development in South Africa (Spire *et al*, 2016; Phaswana *et al*, 2013).

1.2. Health burden of DM and HTN as major NCDs in South Africa

The two most common NCDs associated with rapid mortality increase are diabetes mellitus (DM) and hypertension (HTN). They frequently occur in the same individuals in clinical practice. The presence of HTN does increase the risk of new-onset of DM, as well as DM does promote the development of HTN (Mohan *et al*, 2013; Volpe *et al*, 2015). DM is associated with elevated blood glucose levels whilst HTN is defined as sustaining a blood pressure of ≥140/90 mmHg (Anwer *et al*, 2011; Sowers, 2003; Shah and Afzai, 2013; Suh *et al*, 2009; Grossman and Grossman, 2017). Comorbid HTN and DM are associated with high rates of macrovascular and microvascular complications. DM is commonly accompanied by other CVD risk factors, such as hypertension, obesity, and dyslipidemia. CVDs are the most common cause of death in people with DM (Lorber, 2014).

1.3. Risk factors for DM and HTN

Both these diseases, i.e. DM and HTN are medical conditions also known as non-infectious diseases that result from the combination of modifiable- risk (behavioural) and non-modifiable- (genetic, physiological, and environmental) factors (Alberti *et al*, 2007) (As shown in **figure 1.1**). Hence, in the following section, we discuss these specific risk factors with respect to DM and HTN.

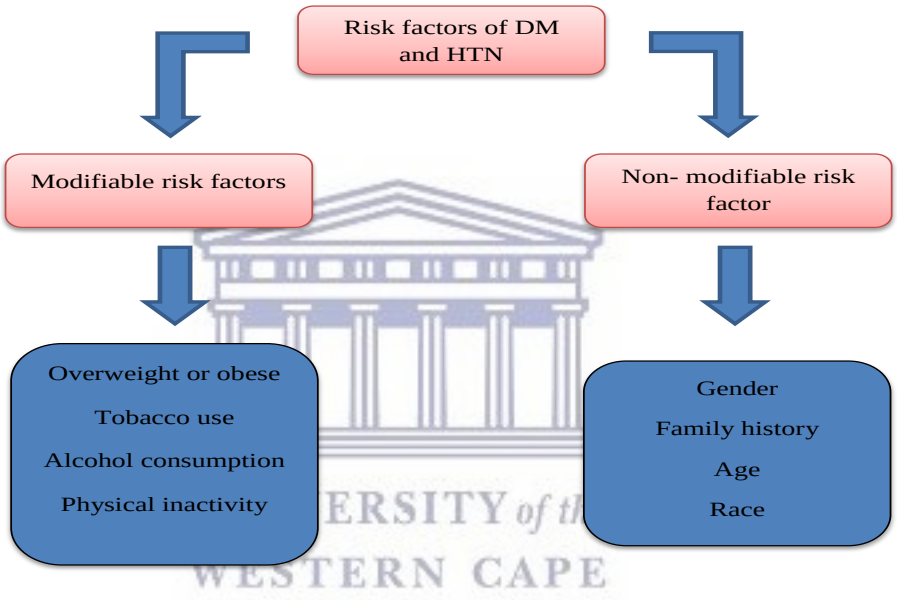


Figure 1.1. Schematic diagram of modifiable and non-modifiable risk factors associated with DM and HTN (Taken from Alberti *et al*, 2007; Issaka *et al*, 2018).

1.3.1. Modifiable risk factors of DM and HTN

Since the majority of studies are done in the western countries a little is known about DM natural history coexisting with HTN and the clinical significance of these studies in African countries (Unwin *et al*, 2001; Levitt, 2008). In South Africa, about 56% of individuals between the ages of 15 – 64 years are assumed to have at least one modifiable risk factor for chronic diseases of lifestyle. These specific

lifestyle factors play an important role in the occurrence of DM and HTN. DM and HTN are often linked to an increase in age and their coexistence increases in elderly individuals (Alberti *et al*, 1998; Wilson *et al*, 2005; Conen *et al*, 2007). The behavioral risk factors for both these diseases include unhealthy diet, obesity, physical inactivity, tobacco use, and harmful consumption of alcohol (Steyn *et al*, 1997; Bradshaw, n.d; Alberti *et al*, 2007), and in terms of disability-adjusted life years (DALYs), attributable are associated with NCDs (Feigin, 2016).

Overweight and obesity are risks factors which are linked to all four major NCDs (i.e. CVD, cancer, DM and chronic respiratory diseases) (Kim and Oh, 2013). In addition, obese people are also at risk of contracting HTN, although the majority of studies on DM (Issaka *et al*, 2018) and being overweight and/or obese increases the development of insulin resistance and progression of the disease. Furthermore, WHO indicated that almost 90% of patients with DM and HTN have excess body weight (He *et al*, 2009b; Davy and Hall, 2004). In the sub-Saharan African countries, South Africa is observed to be the top one with the highest rate of overweight or obesity, with nearly 70% women and 39% men who are overweight (Ng *et al*., 2014). Recent studies have however shown the level of overweight and/or obesity in men also increase (Ardington and Case, 2009). While the overall level of being overweight and /or obese in South Africa is observed to be high in women as compared to men and with approximately 68% of women above 35 years old are overweight or obese based on body mass index (BMI) rate. BMI is an independent risk factor for both DM and HTN, defined as a measure of dividing weight by height squared and commonly used in the overweight and obese

classification in adults (National Department of health, 2017; Cois and Day, 2015: Issaka *et al*, 2018).

Furthermore, Unhealthy diets include sugars, salts, and fat intakes are associated with CVD (risk factor is HTN), DM and other types of cancers. These diets include overweight and /or obesity that are associated with elevated high blood pressure, bad cholesterol, and resistance to the action of insulin. South Africans have shifted from traditional diets such as stable grains, vegetables, and fruits to the western diet which is cheap, energy-dense and nutritional-poor and processed foods. Furthermore, it has been observed that by the next coming years "western diets' will have been used across the world leading to an increased level of unhealthy diets (Bourne *et al*, 2002; Igumbor *et al*, 2012; World Cancer Research Fund International and The NCD Alliance, 2014; Kunene and Taukobong, 2017).

Unhealthy diets are often linked with poor physical activity. Regardless of strategies or attempts to decrease physical inactivity level, only a few adults and children in high-income countries engage in physical activity to maintain or improve health and physical well-being. Physical inactivity is associated with HTN and it is also a known risk factor for obesity and diabetes (Church, 2011; Kruger *et al*, 2005). Blood cholesterol and blood pressure are maintained with physical activity that leads to reduced cholesterol improving the low-density lipoprotein (LDL) to high-density lipoprotein (HDL) cholesterol and lowering triglycerides (Qi *et al*, 2008; Asif, 2014; Colberg *et al*, 2010; Kwon and Lee, 2017). Therefore, healthy diets and physical activity are less likely to be at the risk of contracting

chronic diseases. Moreover, the traditional diets which were used in the past decades must be implemented back.

Alcohol consumption and tobacco use also play a role in the development of DM and HTN, and they are both are risk factors that can be easily prevented. The consumption of alcohol results in an increased risk of chronic liver disease, heart failure and certain types of malignancies (Mukong *et al*, 2017; Ezzati and Riboli, 2013). Whilst, Tobacco use leads to lung cancer, heart diseases, renal failure, and stroke. Implementations and strategies control legislation have been comprehended resulting in a reduction in tobacco use (department of health, 2004a; Shisana *et al*, 2013; Reddy *et al*, 2015). The same decline has been observed for also consumption of alcohol as indicated for tobacco use. Recently, the alcohol per capita consumption is 6.4 liters per person and expected to be 7.0 liters increase in 2025 worldwide unless the strategies to reduce the prevalence of alcohol consumption are successful (WHO, 2018b).

Moreover, in South Africa, alcohol consumption is approximately 5 million liters of alcohol annually, equivalent to 9-10 liters pure alcohol per person (Seggie, 2012) and tobacco use is estimated to kill 44000 South Africans every year (Teare *et al*, 2018; Drope *et al*, 2018). The evaluation of these risk factors indicates the need for lifestyle changes that could lead to a reduction in morbidity and mortality caused by NCDs. This gives concerns about the prevalence of heavy episodic drinking and tobacco use in South Africa.

1.3.2. Non-modifiable risk factors of DM and HTN

Non-modifiable risk factors contribute to the global burden of NCDs and are risks factors that cannot be reduced or controlled by intervention age, gender, ethnic background and family history (genetics). The risk of developing DM and/or HTN is significantly high when one of your family relatives is diabetic or hypertensive. In numerous family studies, the hereditary nature of these diseases is well established (Ranasinghe *et al*, 2015; Manning *et al*, 2016). Generally, DM and HTN occur in middle-aged adults, most frequent 45 years of age. However, recently health practitioners are diagnosing more and more children and adolescents with DM. Moreover, the older a person grows, the more likely are to suffer from heart disease and DM. Although all the risk factors mentioned cannot be changed, they can be managed early by educating people about a healthy lifestyle. According to a study done by Mass and Appelman, (2010), states that males are at higher risk of heart disease than female until the age of 75 although in other studies the difference remains the same (Maas and Appelman, 2010; Bots *et al*, 2017). These factors are important as they also affect prevention and treatment approaches towards the burden of NCDs (Manning *et al*, 2016) and these factors must be targeted at the early stage in order to reduce NCDs (Darnton *et al*, 2004; Singh *et al*, 2017).

1.4. Diabetes mellitus

Diabetes mellitus (DM) is a group, which is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Association, 2010). DM can lead to multiple diseases such as coronary artery disease, impairment of physical and cognitive function and/or death (Mohan *et al*, 2013;

Alwan, 2011). There are two main types of DM; type 1 diabetes mellitus (T1DM) described as the inability to produce insulin, while type 2 diabetes mellitus (T2DM) produced insulin is not sufficient for its function, mainly due to environmental and lifestyle risks factors (Dostalek *et al*, 2012, Olokoba *et al*, 2012). It was previously described that a healthy lifestyle and physical activity can prevent the development of T2DM (Asif, 2014, Chasan-Taber, 2015).

Microvascular and macrovascular complications are associated with long term complications of DM (Stratton *et al*, 2000; IDF, 2009). Diabetic patients with microvascular complications suffer from neuropathy leading to damage of the nervous system, nephropathy leading to chronic renal damage and retinopathy resulting to loss of vision leading to blindness (Stratton *et al*, 2000; Fong *et al*, 2004; Silva *et al*, 2017; Beckman and Creagar, 2016). Microvascular complications lead to mortality in diabetic patients with CVD, stroke and vascular peripheral disease (Silva *et al*, 2017; Beckman and Creagar, 2016). In addition, the risk of the macrovascular disease under the diagnosis of DM with hyperglycemia has already been increased. Therefore, the identification of factors contributing to these complications enables these complications to be controlled and can lead to a significant reduction in morbidity, mortality and cost of health care.

1.5. Type 2 Diabetes Mellitus (T2DM): Epidemiology and Pathophysiology

The prevalence of T2DM is increasing at an alarming rate globally. Because of this trend, it is rapidly becoming an epidemic in some countries of the world with the number of people affected to double in the next decade or years (WHO, 2016). T2DM accounts for major cases (90-95%) of diabetes (Dastalek *et al*, 2012; Pollastro *et al*, 2015; Topić, 2014; Cook *et al*, 2007) and is the major cause of renal failure, obesity, stroke and cardiovascular disease (Todd and Florez, 2014). Moreover, it is one of the leading causes of morbidity and mortality, consuming a significant proportion of public health expenditure.

1.5.1. Epidemiology of T2DM

At present, 422 million people are diagnosed with DM and this number is estimated to reach 642 million by the year 2045. In the year 2010, an estimated 316 million people had impaired glucose tolerance and are at high risk from the diseases. Approximately 200 million people worldwide are affected with T2DM, including more than a quarter of elderly living in developed countries (Lorenzati et al, 2010). Due to population growth, aging, urbanization and increased prevalence of obesity and physical inactivity, the number of people with DM is increasing and about 5 million people died of DM in 2015 globally (Steyn *et al*, 1997; Kengne *et al*, 2013; Peer *et al*, 2014; Ogurtsova *et al*, 2017). In the year 2009, DM was estimated to have caused approximately 8000 blindness and 2000 amputation cases of each year worldwide (Bertram *et al*, 2013). Moreover, the prevalence of DM in men is higher than in women, but more women have DM than men (Wild *et al*, 2004; WHO,

2016). In the African region, an estimated 15.5 million adults between 20 and 79 years of age have DM, representing a regional prevalence of 2.1- 6.7% (IDF, 2017). Some of the most populous countries in Africa have the highest number of DM patients, including South Africa (1.8 million), Democratic Republic of Congo (1.7 million), Nigeria (1.7 million) and Ethiopia (2.6 million) (IDF, 2009). DM indicates a significant burden on the South African health system due to its association with several microvascular and macrovascular complications (Pheiffer *et al*, 2018).

1.5.2. Pathophysiology for T2DM

Defective insulin secretion is central to the pathophysiology of T2DM. To maintain normal glucose levels, insulin secretion varies over a wide range in response to insulin sensitivity (Skyler *et al,* 2017). In general, plasma glucose levels in a narrow and well-balanced range known as homeostasis are maintained. Physiological conditions usually occur in this way; glucose from the diet or carbohydrate breakdown in the intestine and absorbed into the bloodstream (Shah *et al*., 2000) leading to an increase in blood glucose. This triggers insulin secretion from the pancreatic β cells by binding to specific receptors and facilitating the entry of glucose from the blood into the cells. The cells then use glucose for energy resulting in a decrease of blood glucose level (Shah *et al*, 2000).

There are mechanisms that lead to insufficient insulin production resulting in secretion by destroying pancreatic β cells. This influences insulin secretion and releases less insulin on demand, which is called insulin resistance which at the end lead to the development of T2DM (Holt, 2004; Baynest, 2015; Zaccardi *et al*, 2016;

Lee and Halter, 2017; Jin, 2009). It causes an imbalance in glucose production and glucose intake (Lee and Halter, 2017; Shah *et al*, 2000; Boden, 1996) and this result in hyperglycemia (as shown in **Figure 1.2**). Nevertheless, hyperglycemia itself can damage pancreatic β-cell function and worsen insulin resistance, leading to hyperglycemia malicious cycle that exacerbates the metabolic state (Yki-Järvinen, 1992; Li *et al*, 2004).

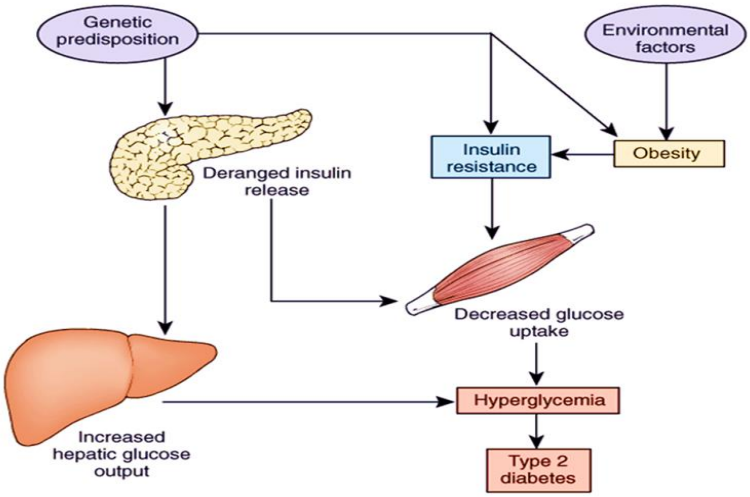


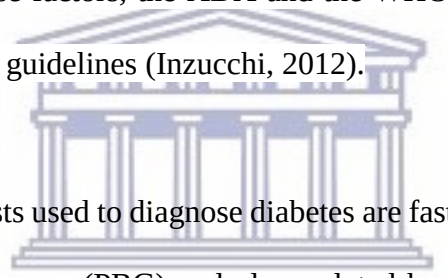
Figure 1.2. Pathophysiology of T2DM (Belleza, 2016).

A number of genetic, environmental and behavioral risk factors are responsible for this problem. Thus, insulin resistance results in peripheral tissue, mainly liver, muscle and adipose (Holt, 2004; Baynest, 2015; Zaccardi *et al*, 2016; Lee and Halter, 2017; Jin *et al*, 2009). In addition, there are also several counter-regulatory hormones known to increase blood glucose include glucagon, growth hormone, catecholamines and glucocorticoids. This could lead to major comorbidities, such as CVD and stroke (Mealey and Ocampo, 2007).

1.6. Drug treatment of type 2 Diabetes Mellitus (T2DM)

1.6.1. Diagnosis of T2DM

Diagnosis of T2DM is carried out on the basis of guidelines provided by the American Diabetes Association (ADA) and/or the World Health Organisation (WHO). These guidelines are consistent with single raised glucose reading, although in patients with microvascular complications with the following classic hyperglycaemia symptoms (polyuria, polydipsia, blurred vision, fatigue and weight loss) and these complications are to be noted when patients are diagnosed with T2DM (Cox and Edelman, 2009; Freeman and Cox, 2006; WHO, 2006; Kumar *et al*, 2016). Due to these factors, the ADA and the WHO lowered the diagnosis of T2DM recommended guidelines (Inzucchi, 2012).



The most common tests used to diagnose diabetes are fasting blood glucose (FBG), postprandial blood glucose (PBG) and glycosylated hemoglobin (HbA1c). These diagnostic tests have recommended diagnostic cut point criteria for FBG (5.6-6.9 mmol/L), PBG (>7.8 mmol/L) and HbA1c (≥6.5%) (SEMDSA, 2017), furthermore, WHO criteria cut point is slightly different to the ADAs (American Diabetes Association, 2015). The first diagnostic tests are the glucose and urinal test that determines whether glucose is present. This criterion for diagnosis is applied not only to adults but also to children (Emancipator, 1998). Healthy patients who have one or more risk factors such as obesity, HTN and family history of DM should be screened (Inzucchi, 2012; WHO, 2015c).

1.6.2. Major classes of anti-diabetic drugs

The initial therapies recommended to prevent T2DM at an early stage are dietary and lifestyle modifications, yet the use of oral antidiabetic drugs plays an important role. These interventions and pharmacotherapy lead to the maintenance of glucose control and prevention of complications associated with disease (Sherifali *et al*, 2010). The major classes of oral anti-diabetic drugs (OADs) include sulfonylureas (SUs), thiazolidinediones (TZDs), biguanides, meglitinides, insulin and glucose-like peptide (Inzucchi *et al*, 2015).

1.6.2.1 Sulfonylureas (SUs*)*

Sulfonylureas (SU) are one of the most widely used classes of oral hypoglycemic agents (Topić, 2014). These SU drugs include Glibenclamide, tolbutamide, glimepiride, and gliclazide (Topić, 2014). Even with good efficacy, these drugs were associated with a number of side effects, such as weight gain and hypoglycemia risk (Klein *et al*, 2014; Pollastro *et al*, 2015).

SUs drugs can increase the insufficient production and secretion of insulin. SUs enhance the release of insulin from pancreatic β-cells by binding the plasma membrane sulfonylurea receptor I (SURI) to the ATP k+ channel (KATP), which leads to the closure of the potassium channel on the β-cell islet. The inhibition of potassium efflux and the depolarization of the plasma membrane leads to the opening of channels of voltage gate (Shyng and Nichols, 1997; Semiz *et al*, 2013; Dawed *et al*, 2016). Resulting in the influx of calcium and a corresponding increase in intracellular calcium levels causing insulin to be released from β-cells. KATP is

a heterooctamers protein complex assembled from potassium inward rectifier 6.2 (Kir6.2) subunit and SUR1, encoded by KCNJII (potassium inwardly-rectifying channel, subfamily J, member II) and ABCC8 (ATP-binding cassette transporter sub-family C member 8) genes respectively (Semiz *et al*, 2013; Li *et al*, 2014; Dawed *et al*, 2016).

1.6.2.2. Thiazolidinediones (TZDs)

Thiazolidinediones (TZDs), also known as glitazones, are an anti-diabetic drug that acts by activating their molecular target, nuclear peroxisome proliferator-activated receptors (PPARs) (Topić 2014). They have been synthesized to minimize the side effects of other diabetic drugs. TZDs improve patients ' insulin sensitivity and reduce hyperglycemia by reducing the fatty acid concentration and lipid access in the liver and muscles. The main side effects of TZDs include hypertension, hepatic steatosis and microalbuminuria (Karalliedde and Buckingham, 2007). The approved TZD drugs include troglitazone, pioglitazone, and rosiglitazone. However, troglitazone was removed from the market due to severe hepatotoxicity (Topić, 2014; Dawed *et al*, 2016). TZDs exact mechanisms are unclear, however, they primary activate PPARγ target in adipose tissue and affects glucose and lipid metabolism. (Topić, 2014; Semiz *et al*, 2014; Pollastro *et al*, 2015)

1.6.2.3. Biguanides

The Biguanides class of anti-diabetic drugs includes metformin, phenformin, and buformin. Buformin was introduced for the first time in 1958, while metformin (Glucophage) and phenformin were made available only in1975 (Bailey, 1992;

Bastaki, 2005). Both phenformin and buformin were discontinued in 1970 due to toxicity and high incidence lactic acidosis. Although the structure of metformin is more like phenformin (**Figure 1.3**), it has a very low incidence of lactic acidosis in diabetic patients (Sambol *et al*, 1996; Powers and D'Allesio, 2011; Tarasova *et al*, 2012; Wang *et al*, 2003). These drugs are developed from *galegine*, a guanidine derivative found in *Galega Officinalis*. Metformin was approved by FDA (Food and Drug Administration) in 1994 (Cruzan, 1994; Goodarzi and Bryer-Ash, 2005) and it was recommended to be used as first-line drug therapy in treatment of diabetes by International Diabetes Federation (IDF) and the American Diabetes Association and European association, and by researchers despite their adverse effects (Reitman and Schadt, 2007; Todd and Florez, 2014).

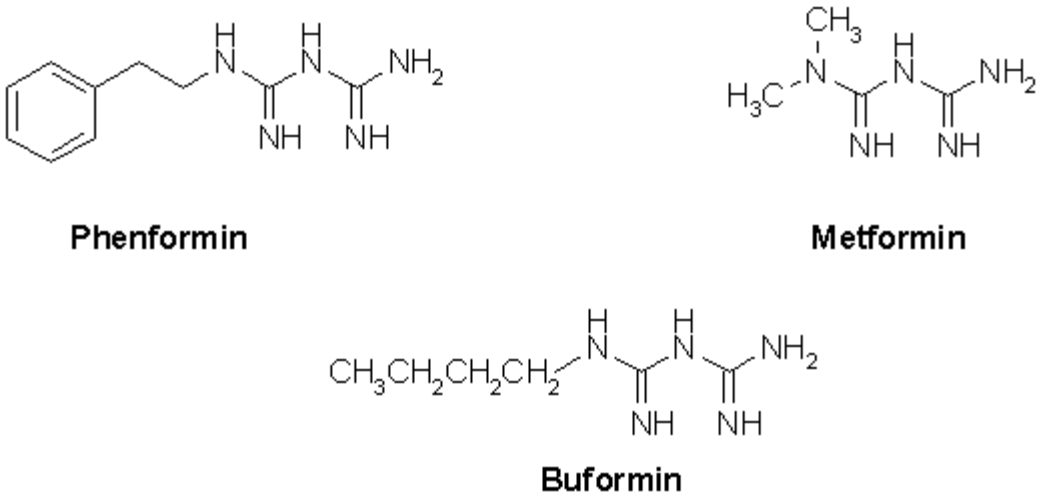


Figure 1.3. Representative of the biguanides antidiabetic class: the chemical structures of the biguanides drugs (Desai, 2000).

1.7. Metformin as a first-line anti-diabetic drug

Metformin does not have any chemical or pharmacological relationship with any other class of oral antihyperglycemic agents (Ramanjireddy *et al*, 2011). It is a small water-soluble basic compound (molecular weight=129Da), which exists in physiological pH as an organic cation and has an insignificant binding to plasma proteins (Kovo *et al*, 2008; Graham *et al*, 2011). Metformin has been recommended as a T2DM first-line drug for over than 20 years, although its mechanism is tentative (Bailey, 1996; Shu *et al*, 2007). However, it is known that its mechanism of action is by indirectly activating AMPK (AMP-activated protein kinase) via the upstream kinase regulator (Viollet *et al*, 2012; Todd and Florez, 2014, Zhou *et al*, 2001). It is actively transported by organic cation transporters (OCTs), plasma membrane monoamine transport (PMAT) and multidrug and toxins extrusion protein (MATE) (**Figure 1.4**) (Du Plessis *et al*, 2015; Becker *et al*, 2009b; Shikata *et al*, 2007).

Metformin is used to treat hyperglycemia by improving the sensitivity of insulin while reducing hepatic gluconeogenesis (Violette *et al*, 2012; Goswami *et al*, 2014; Chen *et al*, 2015a). Furthermore, it has beneficial effects on cardiovascular diseases, impaired glucose tolerance and insulin-sensitizing polycystic ovary syndrome and resulting in also less weight gain (Kirpichnikov *et al*, 2002; Janci *et al*, 2012). Metformin has an advantage over sulfonylurea; it does not lead to hypoglycemia or hyperinsulinemia as monotherapy in T2DM or healthy patients. However, the most common adverse effects such as gastrointestinal and lactic acidosis have been reported (Amod, 2012; Powers and D'Allesio, 2011) and these effects usually occur in the early stages of treatment.

1.7.1. Gastrointestinal and Lactic acidosis adverse effects

There have been frequent reports on gastrointestinal side effects resulting from metformin therapy. Gastrointestinal side effects include diarrhea, abdominal pain, flatulence and bloating (Defronzo, 1999; Haupt *et al*, 1991; Tarasova *et al*, 2012; Fatima *et al*, 2018; Du *et al*, 2018). Though these side-effects are often transient and can be managed by reducing the dosage used and patients are also advised to administer the drug with meals, though, about 5% of these patients turn to recur even at a lower dosage (Cusi and DeFronzo, 1998; Hermann, 1979; Mkele, 2013). The accumulation of metformin during treatment could also cause serious metabolic complications such as lactic acidosis. The latter complication is characterized by anorexia, vomiting, abdominal pain, thirst, and nausea. Lactic acidosis associated with metformin is very low and it is fatal in approximately 50% of cases when it occurs (Papanas and Maltezos, 2009). It usually occurs due to overdose or in some contraindicated condition such as cardiovascular collapse, acute myocardial infarction, congestive heart failure or chronic metabolic acidosis, including diabetic ketoacidosis and liver dysfunction (Mkele, 2013).

1.8. Metformin Pharmacodynamics and Pharmacokinetics

1.8.1. Drug transporters

Membrane transporters are a class of membrane proteins found in all organisms, responsible for cell homeostasis sustainability and may be key determinants of the safety, efficacy and pharmacokinetic profile of a drug. They control the influx of essential nutrients and ions and the efflux of cellular waste, xenobiotic and environmental toxins (Giacomini *et al*., 2010). Membrane transporters can

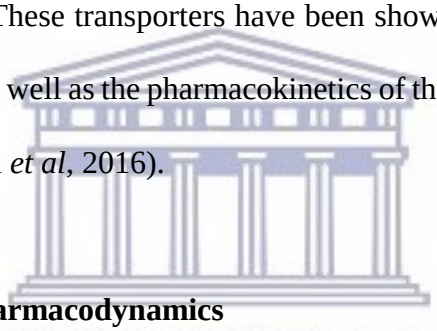
potentially contribute to drug permeability in cells and to drug access to their pharmacological and toxicological targets (Russel, 2010). Over the last 20 years, a large number of membranes have been identified as therapeutic goals in the treatment of various types of diseases such as DM, major depression, HTN and constipation, etc. (Liang *et al*, 2015; Brockmoller and Tzetkov, 2008). Membrane transporters are also drug resistance determinants and are important in the absorption, distribution and elimination of compounds as transportation- mediated drug-drug interaction (DDI) (Kushuhara and Sugiyama, 2009; Müller and Fromm, 2011; Russel, 2010).

Drug-drug interaction is defined as the combination of two or more drugs in which one drug has a high efficiency because of the presence of the other drug (Liang *et al*, 2015). Possible DDI sites that may influence the pharmacokinetics profile are 1) gastrointestinal absorption, 2) binding of plasma and/or tissue proteins, 3) carrier-mediated transportation across plasma membranes and 4) metabolism. Drug interaction results in adverse effects in patients and is caused by changes in absorption, distribution, metabolism and elimination (ADME) (Liang *et al*, 2015).

Both the kidney and the livers are responsible for drug and xenobiotic elimination (Evans and McLead, 2003; Meyer Zu Schwabedissen *et al*, 2010). It was also found that the intestine, liver and kidney are the main organs that determine drug absorption, distribution and elimination. The regulation of the absorption, distribution and excretion of many medicines is said to play an important role in protein transport. These medicines are actively secreted through a two-step

membrane transport process involving separate systems on the epithelial cell brush and basolateral membranes (Kusuhara and Sugiyama, 2009; Russel, 2010). The carriers in human organisms are the largest family of these transporter proteins; ATP - binding cassette and solute carrier transporters (SLCs). These proteins are among the most widely studied transporters involved in the disposition and effect of drugs (Evans and McLead, 2003).

In the case of metformin, the main drug transporters involved in the transport of this drug include the organic cation transporters (OCTs) and multidrug and toxin extrusion (MATEs). These transporters have been shown to directly influence the pharmacodynamics as well as the pharmacokinetics of the drug Becker *et al*, 2009b; He *et al*, 2015; Shokri *et al*, 2016).



1.8.2. Metformin pharmacodynamics

As mentioned above, metformin mainly involves the suppression of excess glucose production by reducing gluconeogenesis, reducing basal and postprandial plasma glucose (PPG) (Hundal *et al*, 2000). It stimulates glucose intake, insulin signaling, reduces the production of fatty acids and triglycerides and β-oxidation of fatty acids. However, its molecular mechanism of action remains unknown. Researchers have explained the mechanisms by phosphorylation and activation of AMPK in the liver when metformin is administered. AMPK is known as a major lipid and glucose metabolism regulator and is activated via the liver kinase B1 (LKBI; also known as Serine/Threonine Kinase 11 - STK11) kinase regulator. LKBI activation of AMPK is not direct targets of metformin yet metformin direct target is not fully explained.

Furthermore, metformin reduces ATP synthesis in some studies and inhibits the mitochondrial respiratory chain complex I, which eventually leads to AMPK activation by increasing the cellular ratio of adenosine monophosphate (AMP): adenosine phosphate (ATP) (Todd and Florez, 2014; Foretz and Viollet, 2011; Hardie, 2007; Zhou *et al*, 2001). Moreover, the activation of AMPK in other studies leads to increased uptake of glucose by stimulation of glucose transporter type 4 (GLUT4) (encoded by gene SLC4A2) translocation activity (Todd and Florez, 2014; Shu *et al*, 2007).

1.8.3. Metformin pharmacokinetics

Metformin is a hydrophilic organic cation drug; it serves as a substrate for drug transporters (Distefano and Watanabe, 2010; Graham *et al*, 2011). The PMAT (encoded by *SLC29A4*)-transports metformin in the intestine lumen as the membrane is made up of lipid and it is taken up by OCT1 (encoded by *SLC22A1*) and OCT3 (encoded by *SLC22A3*) into the hepatocytes (Zhou *et al*, 2007; Todd and Florez, 2014). Both OCT1 and OCT3 drug transporters are expressed on the basolateral membrane of hepatocytes. In the kidney, metformin is not fully absorbed or taken up in the cell through renal epithelial cells by OCT2 and excreted into the urine via MATE 1 (*SLC47A1*) and the MATE 2 (*SLC47A2*) (Todd and Florez, 2014). OCT2 (encoded by *SLC22A2*) regulates the accumulation of metformin in the basolateral membrane kidney and plays a major role in the extraction of metformin into urine via tubular secretions (Roth *et al*, 2011; Tarasova *et al*, 2012). MATE 1 and MATE 2 facilitates metformin excretion from

hepatocytes and renal epithelium into bile and urine, respectively (Distefano and Watanabe, 2010 Kimura *et al*, 2005a; 2005b) (**Figure 1.4**).

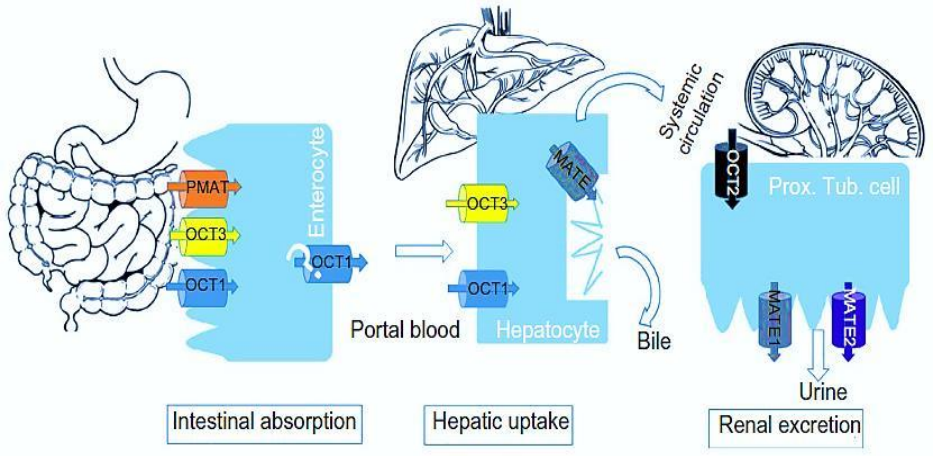


Figure 1.4. Schematic diagram indicating OCT and MATE transporting route. Drugs are digested into the intestine where they are transported by the Plasma Membrane Monoamine Transporter (PMAT) into the enterocyte. OCT1 transports the drug out of the intestine into the hepatocyte. OCT1 and OCT3 transport the drug(s) out of the intestine into the hepatocytes. MATE1 and MATE2 transport the drug(s) from the hepatocytes into the kidney towards the tubular ducts (bile and urine) for excretion, which facilitated *by* OCT2 (Dawed *et al*, 2015).

1.9. Pharmacogenomics of metformin response in T2DM

1.9.1. Variability in metformin response in T2DM.

As previously described, metformin is the first line drug prescribed to T2DM patients (Wang *et al*, 2003; Gong *et al*, 2012). Despite its excellent efficacy and safety profile, about 30-40% of these patients who have taken metformin failed to reach the fasting glucose level (He *et al*, 2015; Huang and Florez, 2011). Research suggests that this variation in response to metformin treatment can be attributed to the presence of SNPs in the drug targets, drug transporters and metabolizing

enzymes (Avery, 2009, Mashahit *et al,* 2014). Thus, these SNPs are responsible for inter-individual variability in pharmacokinetics, efficacy and therapeutic drug toxicity (Du Plessis *et al,* 2015; Umamaheswaran *et al*, 2014). Genetic variations in drug transporters are increasingly being recognized as a possible mechanism that can explain the inter-individual variability in drug efficacy and toxicity (Shu *et al*, 2007). Information of SNPs commonly associated with metformin therapy response, including those listed in **Table 1.1**., is available on the Pharmacogenomics Knowledge Base [PharmGKB (http: //www.pharmgkb.org)], and other specialized databases, as well as the literature.

1.9.2. Pharmacogenomics Knowledge Base (PharmGKB)

The Pharmacogenomics Knowledge Base (PharmGKB) is a Web-based database that aims to aid researchers in understanding how genetic variation among individuals contributes to differences in reactions to drugs (Whirl-Carrillo *et al*, 2012). The level of evidence score consists of several criteria such as replication of the association, *P* value and odds ratio, if available. The criteria's are divided into four levels (1-4), level 1 and 2 are divided into A, and B subtypes. In level 1, the annotation involves a variant-drug combination in which the majority of evidence shows an association of the patient drug response (Whirl-Carrillo *et al*, 2012). The association must be replicated in more than one cohort with significant *P* values and, preferably, with a strong effect size. In level 2, the annotations are for variant–drug combinations with moderate evidence of an association. The association for level 2 annotations must be replicated but may include negative studies as well (Whirl-Carrillo *et al*, 2012). In level 3, annotations are based on a single significant

(not yet replicated) study or annotation for a variant-drug combination evaluated in multiple studies but lacking clear evidence of an association. For level 4, the annotations are based on a case report; on a study that did not achieve significance but is biologically plausible; or in vitro, molecular, or functional assay evidence (Whirl-Carrillo *et al*, 2012).

1.9.3. Association of genetic variants and metformin response

Numerous studies investigated the associations between the genetic variants of OCTs and MATEs drug transporters and the clinical pharmacokinetics or efficacy of metformin. Furthermore, only a few studies explore genetic variation for metformin pharmacodynamics, which likely reflects the uncertainty surrounding the molecular mechanism of response (Todd and Florez, 2014).

Previous studies have shown that the non-synonymous variant in the *SLC22A1* gene is highly polymorphic and affects its functionality (Nies *et al*, 2011; Todd and Florez, 2014). A recent study by Umamaheswan *et al*. (2014) investigated the influence of genetic variation in *SLC22A1*; an rs622342 variant on metformin response in T2DM patients in South India, and has shown that it was a significant modulator in metformin response (Umamaheswaran *et al*, 2014). In another study, this variant was found to be associated with a blood glucose lowering effect of metformin (Becker *et al*, 2009b). A larger cohort of studies is needed to validate what was predicted by Umamaheswaran *et al*. (2014); Becker *et al*. (2009b), on an rs622342 variant with regards to the association to metformin response. This variant was reported to influence the pharmacodynamics response of metformin suggesting

a reduction in transport activity associated with a higher reduction in HbA1c (Meyer Zu Schwabedissen *et al*, 2010). However, in attempting to replicate the finding of Becker *et al*. (2009b) to a small cohort of the Rotterdam study, no association was found between this variant and change in HbA1c after 6 month's therapy (Becker *et al*, 2009b; Tkáč *et al*, 2013).

Becker *et al*, (2009b) identified that the rs22899669 variant reduces the functioning of *SLC47A1*, delaying transport and elimination of metformin and eventually improving the glucose-lowering effect (Becker *et al*, 2009b). However, Tkáč *et al.* (2013), found it difficult to elucidate the effect of this variant on the mechanism for metformin action since the *SLC47A1* rs22899669 is a non-coding intronic variant (Tkáč *et al*, 2013). He *et al*. (2015) identified that this non-coding variant (rs22899669) is in linkage disequilibrium with more than one SNP. These findings could be important in future treatment for T2DM. In addition to this, the variant rs594709 has been shown to influence the therapeutic efficacy of metformin in lowering glucose levels, decreasing serum lipid and improving insulin sensitivity (Xiao *et al*, 2016). Moreover, several previous studies have also reported an association between metformin pharmacokinetics and an intronic variant of MATE1 rs2289669 and promoter variants of MATE-2K (rs34834489, rs12943590) (Stocker *et al*, 2013, Becker *et al*, 2009b; Chung *et al*, 2013).

Variant rs12943590 was shown to be a significant associated with poor glycaemic response to metformin in newly diagnosed T2DM patients (Choi *et al*, 2011). Moreover, in other studies, it was also shown to be associated with reduced

metformin response in diabetic patients due to pharmacokinetics mechanism and pharmacodynamics (Shocker *et al*, 2013; Christensen *et al*, 2013). Another SNP in OCT2 (rs316019) was shown to be associated with reduced renal and secretory clearance of metformin (Tarasova *et al*, 2012). Other SNPs investigated in this study were also associated with metformin side effects (Tarasova *et al*, 2012).

A role of OCT3 was studied by Chen *et al*. (2010c) and identified six non-synonymous variants through sequencing analysis and only three were identified (i.e T44M (rs8187715), T400I (rs8187725) and V423F (1267G>T) to be significantly associated with the altered response of metformin action (Chen *et al*, 2010c). Moreover, there are candidate gene studies that report an association with metformin response and efficacy. A genome-wide approach in assessing variants involved in metformin was hypothesized. The GoDARTS and UKPDS groups conducted an investigation on the genetics of metformin response in Scottish individuals with T2DM and incident metformin use. The ATM (ataxia telangiectasia mutated) candidate gene has an SNP rs11212617 that was identified to have a potential to achieve HbA1c level>7% by the UK participants with T2DM (Van Leeuwen *et al*, 2012). Nevertheless, other variants have been reported to be associated with the function reduction in both in *vivo* and *vitro* in OCT1 (Christensen *et al*, 2015). Even though, some studies show a contradictory for the association between the *KCJII* variant and SU response in combination with metformin. This variant has been shown to have a significantly increased risk failure in the SU response. In other studies, no evidence was observed of the

associated of SU with a decrease in HbA1c for long-term treatment (Klein *et al*, 2014).

Only a few studies have examined the influence of variants in FMO (1-6) on metformin response and effectiveness. The FMO (Flavin-containing monooxygenase) group is an example of the metabolizing enzymes focused on in the study. FMO is nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) dependent flavoenzymes that catalyze the oxidation of nucleophilic nitrogen, sulfur and phosphorus atoms in a wide variety of drugs and/or pesticides. Three enzyme forms, *FMO1* found in fetal liver, *FMO2* in adult liver, and *FMO3* (located in the liver) are encoded by chromosome- clustered genes 1q23-q25 (Ziegler, 2002; Van Berkel, 2005; Phillips *et al*, 1995). *FMO4* and *FMO5* are expressed in various tissues, whilst *FMO6* is mostly located in kidneys, heart, testes, uterus, and liver (Uehara *et al*, 2017).

However, *FMO5* exhibits similar catalytic activities in rabbit, guinea pig and human *FMO5* enzymes, which are distinct from those of other FMOs. It is not considered as a drug-metabolizing enzyme in contrast to other forms but it is a paralog gene derived from *FMO2*. Among its related pathways are metabolism of xenobiotic by cytP450 and pharmacodynamics (Overby *et al*, 1995; Dolphin *et al*, 1998). Breitenstein *et al*. (2015), identified *FMO5* appears to be marginally significantly associated with decreases in glycemic response after exposure to metformin, representing an EHR-driven pharmacogenetics hypothesis that could represent a

novel mechanism for the biotransformation of metformin that has been previously unidentified (Breitenstein *et al*, 2015)

Table 1.1 Information of SNPs commonly associated with metformin therapy response.

SNP ID	Amino acid change	Gene	Types of gene	Drug	Clinical phenotype	Type	Level of evidence [a]	References
rs10213440	30362A-G	*PPARGCIA*	-	Metformin	-	Efficacy	-	Tkáč *et al*, 2015
rs11212617	5285C-A	*ATM* (*C11orf65*)	Serine/ threonine protein kinase	Metformin	DM, T2DM	Efficacy	2B	Van Leeuwen *et*, 2012
rs12208357	R61C	*SLC22A1*	Drug transporter	Metformin	-	Metabolism /PK	3	Shu *et al*, 2008, Dujic *et al*, 2017
rs12943590	130G-A	*SLC47A2*	Drug transporter	Metformin	DM	Efficacy, metabolism/ PK	3	Stocker *et al*, 2013, Chung *et al*, 2013, Christensen *et al*, 2015
rs2617102	4606687A>C	*CSMDI*	-	Metformin	-	Efficacy	-	Breitenstein *et al*, 2015
rs594709	160134722G-A	*SLC22A1*	Drug transporter	Metformin	DM, T2DM	Efficacy	4	Xiao *et al*, 2016

rs784892	10246G-A	*AMHR2*	-	Metformin	DM	Efficacy, metabolism/ PK	3	Goswami *et al* , 2014
rs784888	11870G-C	*SPI*	Protein non-coding gene	Metformin	DM, T2DM	Efficacy, metabolism/ PK	3	Goswami *et al*, 2014, Santoro *et al*, 2018
rs34399035	1285G-A	*SLC47A2*	Drug transporter	Metformin	-	Metabolism /PK	-	Choi *et al*, 2011
rs2282143	Pro341Leu	*SLC22A1*	Drug transporter	Metformin	-	Metabolism /PK	3	Yoon *et al*, 2013
rs12752688	171182499C-T	*FMO2*	Drug metabolizing enzymes	Metformin	-	Efficacy	-	Breitenstein *et al*, 2015
rs1920145	171076659T-C	*FMO3*	Drug metabolizing enzymes	Metformin	-	-	-	Breitenstein *et al*, 2015
rs2076322	133-126A-G	*FMO4*	Drug metabolizing enzymes	Metformin	-	Efficacy	-	Breitenstein *et al*, 2015
rs2076828	698C-G	*SLC22A3*	Drug transporter	Metformin	-	Efficacy	3	Chen *et al*, 2015a

rs2289669	337-158G-A	*SLC47A1*	Drug transporter	Metformin	DM, T2DM, POS	Efficacy	3	He *et al*, 2015, Xiao *et al*, 2016, Tkáč *et al*, 2013
rs34059508	Gly465Arg	*SLC22A1*	Drug transporter	Metformin	-	Dosage, metabolism/ PK	-	-
rs5219	Lys23Glu	*KCNJII*	-	Metformin/ glibenclamide	DM, T2DM	Efficacy	2A	Sesti *et al*, 2006,
rs7541245	860C-A	*FMO5*	Drug metabolizing enzymes	Metformin	DM	Efficacy	3	Breitenstein *et al*, 2015
rs8187725	T400I	*SLC22A3*	Drug transporter	Metformin	-	Metabolism /PK	4	Chen *et al*, 2010c

The selected SNPs were taken from the following databases: Pharmacogenomics Knowledgebase (http://www.pharmgkb.org), the UCSF-PMT. (http://www.pharmacogenetica.usfc.edu /) database and NCBI-SNP database (http://www.ncbi.nih.gov). POS - Polycystic ovary syndrome. DM – Diabetes Mellitus. T2DM – Type 2 Diabetes Mellitus. a. data of the level of evidence from (Whirl-Carrillo *et al*, 2012).

1.10. Precision medicine and pharmacogenomics profiling systems

Over the past decades, advances in pharmacogenomics have yielded new tools in evaluating the susceptibility and prognosis of disease as well as an unprecedented opportunity to individualize drug therapy (Benjeddou, n.d). New medicines are increasingly targeted to specific patient populations and adding much-needed firepower to the therapeutic armamentarium, particularly in commonly occurring chronic diseases such as cancer, diabetes and rare diseases (Benjeddou, n.d). Our knowledge of genetic contributors to a variable in new and existing drugs has expanded dramatically with the movement towards clinical pharmacogenomics implementation (Johnson, 2013). Hence, pharmacogenomics plays a role in the development of rational means to optimize drug therapy with regards to the genotype of the patient (Aneesh *et al*, 2009). Pharmacogenomics, the core element of precision medicine, is the study of genetic variation and drug response, it has not only facilitated patient treatment outcomes but also increased the understanding at the molecular level of both disease and traditional pharmaceutics (Pirazzoli and Recchia, 2004; Bhathena and Spear, 2008; Eichelbaum *et al*, 2006). The understanding of human genetic findings and inter-individual variations in drug response has recently revealed new opportunities for personalized treatments (Issa, 2007). Precision medicine may be described, broadly, as the tailoring of therapeutics to individual genetic profiles (Rich and Cefalu, 2016). It is a treatment aimed at the needs of a specific patient based on the genetic, biomarker, or phenotypic character (Jameson and Longo, 2015). Genetic variants related to drug ADME has been linked to inter-individual variability in drug efficacy and adverse effects. Hence, the identification of pharmacogenomics biomarkers has the

potential of optimizing treatment for individuals, and base on the new medical care paradigm of precision medicine (Arbitrio *et al*, 2018).

Biomarkers are defined as a characteristic that is measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001; Frank and Mittendorf, 2013). Moreover, biomarkers are used to identify patients who are likely to have an adverse event to a specific drug and help reduce adverse effects such as toxicity (Frank and Mittendorf, 2013). Biomarkers can be generally distinguished in two forms i.e. prognostic and predictive biomarkers (Adelstein *et al*, 2011; Jackson and Sood, 2011). Prognostic biomarkers are used as indicators of disease prognosis where general information is provided about the prospective success of treatment, independent of the use of specific pharmaceutical treatment options. In contrast, predictive biomarkers confer for prediction and monitoring of clinical response in the establishment of patients groups for drug treatment response. Furthermore, predictive biomarkers are divided into three forms i.e. resistant, response and risk biomarkers (Jackson and Sood, 2011).

Currently, biomarkers are used in genetic test kits to screen and monitor patients for variations in genes that influence individual response to drugs. Identifying if a patient carries any of genetic variants will assist in prescribing a personalized drug therapy, decreasing the chances of adverse drug events and increasing the effectiveness of the drug. (Liu *et al*, 2019; Ginsburg and Haga, 2019). Biomarkers are used also in obstetrics and pediatrics such as newborn screening and prenatal

screening and diagnosis and have been shown to be effective in clinical practice (Liu *et al*, 2019; Ginsburg and Haga, 2019). In addition, some pharmaceutical companies report biomarker strategies in research and development aiming to refine drug targets for selected patient populations. They also examine chemical compounds to identify and understand how different forms of enzymes break these compounds down. This leads to avoiding toxicity and improve efficacy when adjusting the dose of a drug based on individual genotype (Hodgson and Marshall, 1998; Pistoi, 2002; Ginsburg and Phillips, 2018).

Pharmacogenomics undoubtedly promotes the development of targeted therapies, as demonstrated by the approval of the several drugs by the FDA and the European Medicines Agency. There are currently a number of genotyping methods namely real-time multiplexed PCR or microarray-based assays in performing genetic screening of known pharmacogenomics biomarkers (Adelstein et al, 2011; Mizzi et al, 2014). This has led to the development of a number of commercial and in-house developed pharmacogenomics' profiling systems, which are currently being used for the optimization of patient drug therapies. The current commercial pharmacogenomics profiling assays include, among others, AmpliChip CYP450 Test™ (Roche Diagnostics, Basel, Switzerland) and Spartan RX CYP2C19 'bedside' (Spartan Biosciences, Ottawa, Ontario, Canada) (Crews *et al*, 2012; Shahin and Johnson, 2013; Arranz *et al*, 2013)

1.10.1. Micro-assay AmpliChip CYP450 Test™ assay and optimization of warfarin treatment

Warfarin is an anticoagulant used for the prevention of thrombosis related complication such as stroke and pulmonary embolism among patients with atrial fibrillation (Wadelius *et al*, 2007; Wigle *et al*, 2017). Patients receiving warfarin anticoagulation require frequent monitoring of blood clotting activity, measured by the prothrombin time (expressed as an international normalized ratio (INR)), particularly in the immediate period after the initiation of warfarin therapy (Musunuru *et al*, 2012; Martin, 2009). Vitamin K epoxide reductase complex subunit 1 (*VKORC1*) is the target enzyme warfarin, which has been significantly associated with warfarin sensitivity and reduced dose requirements. Warfarin has two isomers designated S and R with equal amounts. The more potent one is S-warfarin, which is metabolized principally by *CYP2C9*, whilst the R-warfarin is metabolized by *CYP1A2*, *CYP2C19*, and *CYP3A4* (Crews *et al*, 2012). There are two genes i.e. cytochrome P450 2C9 (*CYP2C9*) and VKORC1 known to be involved in outcomes related to warfarin therapy that accounts for more than one-third of the inter-individual variation in stable therapeutic dosing of warfarin (Issa, 2007; Musunuru *et al*, 2012). The three most important variants shown to have clinical implications for warfarin dosing and prevention of adverse events are the polymorphism of *VKORC1* (rs9923231) and *CYP2C9* (rs1799853, rs1057910) gene.

There is a significant risk associated with warfarin therapy: if the dose is low, it leads to thromboembolism and if the dosage is too high, then it causes bleeding.

The visibility of metabolizing enzyme CYP450 testing was raised substantially with the FDA approval of the AmpliChip® CYP450 test, developed by Roche Molecular Diagnostics, Inc. (Arranz *et al*, 2013). The AmpliChip® CYP450 test utilizes DNA microarray technology to analyze patient genotypes for CYP450 genes *CYP2D6* and *CYP2C19* (De Leon *et al*, 2006). Patients who possess homozygous alleles (wild type) metabolize warfarin completely whilst carriers of the allelic variants have less capacity. The carriers of allelic variants require lower doses when tested (Surendiran *et al*, 2008).

1.10.2. Spartan RX CYP2C19 'bedside' assay and optimization of clopidogrel therapy

Clopidogrel is an oral antiplatelet prodrug prescribed to inhibit blood clots, which can lead to heart attack and stroke. The prodrug is metabolized by *CYP2C19* into the active form (Kitzmiller *et al*, 2011). The *CYP2C19* gene variants i.e. rs4244285, rs4986893, and rs12248560, are known to be associated with increased and decreased response to clopidogrel respectively (Brown and Pereira, 2018). Under clopidogrel pharmacogenomics profiling system, patients who carry homozygous allele variants rs4244285 and rs4986893 are poor metabolizers whereas patients with heterozygous allele are classified as intermediate metabolizers. Both these metabolizers lead to an increased risk of ischemic events, in particular, stent thrombosis and intraprocedural thrombotic events during percutaneous intervention. Poor metabolizers' patients experience reduced effectiveness of the drug at standard dosing and alternative therapy must be considered for these

patients. Patients with wild type alleles confer to the desired metabolism (Brown and Pereira, 2018; Musunuru *et al*, 2012).

The Spartan RX CYP2C19 is bedside DNA test that identifies carriers of the *CYP2C19**2 (rs4244285) gene with the use of buccal swab. The *CYP2C19**2 variants are associated with the poor clopidogrel response. Patients who carry this variant are prescribed with an alternative drug instead of clopidogrel treatment. The Spartan RX CYP2C19 is used to eliminate poor response to treatment (Crews *et al*, 2012; O'Connor *et al*, 2012). Although non-genetics factors such as age, obesity, gender play a part in the effect of the platelets function (Brown and Pereira, 2018) and other studies gave a suggestion that platelets activity testing should be considered in clopidogrel drug therapy. Nevertheless, genotyping may be useful to patients with a high risk of blood clots than the ones at lower risk (Brown and Pereira, 2018). Even though routine CYP2C19 testing it is not recommended, there are challenges with implementing pharmacokinetics guided therapy in clinical practice and no firm recommendations have been established regarding dose adjustments for CYP2C19 status (Amin *et al*, 2017; Knauer *et al*, 2015).

1.10.3 Pharmacogenomics assays to predict response to statins' therapy

Statins are drugs used in lowering the concentration of LDL and prevent major coronary events (Musunuru et al, 2012, Kitzmiller et al, 2016). However, statins are associated with the higher risk of myotoxicity or myopathy with a higher dosage than recommended at the start of treatment (Musunuru et al, 2012; FDA, 2011). Therefore, the development of pharmacogenomics tests, which could predict

response to statin therapy, might have significant clinical implications. Research has shown genomic markers i.e. solute carrier organic anion transporter family member 1B1 (*SLCO1B1*), Kinesin-like family 6 (*KIF6*) and cytochrome P450 3A (*CYP3A*), which might assist in the therapy choice of patients initiating statins. For example, *SLCO1B1* is a predictive marker that is a significant barrier for optimal adherence of statin-related myopathy. Approximately 50% of statin-related myopathy is associated with simvastatin attributed to *SLCO1B1* genetic variant (Canestaro *et al*, 2012, Kitzmiller *et al*, 2016).

Genetic variation in *SLCO1B1* in relation to single dose statin plasma pharmacokinetics have suggested that two nonsynonymous SNPs (rs4149056 and rs2306283), alter statin plasma clearance. Patients with *SLCO1B1* gene variant(s) are more likely to have severe muscle pain and/or weakness when taking statins (simvastatin). Moreover, alternative therapy or less simvastatin dosage is recommended when prescribing to patients with these two SNPs. Another example of a biomarker considered for the statins pharmacogenomics profiling assays is KIF6 (Canestaro *et al*, 2012; Musunuru *et al*, 2012; Gelissen, and McLachlan, 2014), in terms of efficacy and genetic predisposition to possible adverse effects even though that aspect remains difficult (Musunuru *et al*, 2012; Gelissen, and McLachlan, 2014). A recent study done concluded that rs20455 variants in the KIF6 gene influence patient responses to simvastatin and atorvastatin treatment (Ruiz-Iruela, *et al*, 2018). However, FDA has not recommended any test in relation to statins yet, as more studies must be conducted. Larger studies are needed to

determine the effectiveness of pharmacogenomics testing in the type of statin, dose, and concomitant use of other drugs in various patient populations set by.

More evidence is produced on abnormal metabolizers due to high genetic variation in individuals, which leads to a significant risk of ADRs and/or decrease in efficacy (Mukerjee *et al*, 2018). In Africa, precision medicine mechanisms are still at a premature stage. The majority of pharmacogenomics biomarkers with potential are discovered in small sample studies, which often need statistical validation in a large cohort of study. In fact, presently many tests and kits are for research use only (Zanger and Schwab, 2013; Evans and Relling, 1999). In addition, the need to be consideration of ethnic differences in the population in targeted genotyping that results in a limitation of genomic variants selection and missing others in a specific population (Mukerjee *et al*, 2018). For example, rs83495 and rs9035037 is implicated in the response to warfarin therapy and are commonly in Caucasians but not frequent in African-Americans (Li *et al*, 2009). Identifying pharmacogenomics biomarkers will assist clinicians to personalize medical care and point out the best drug and optimal dose for the specific individual (Matimba, 2009 Ph.D. thesis).

1.11. Summary and main objectives of the study

South Africa is one of the countries experiencing an increasing burden of non-communicable diseases (NCDs). NCDs are the major source of mortality and morbidity, which is estimated to surpass the burden of infectious diseases by 2035. The two most common NCDs associated with rapid mortality increase are diabetes mellitus (DM) and hypertension (HTN). They frequently occur in the same individuals in clinical practice. Both of these diseases, i.e DM and HTN, can be a result of a combination of modifiable risk factors (behavioral factors) and non-modifiable risk factors (genetic, physiological, and environmental). The burden of NCDs in South Africa is predicted to increase substantially in the next decades if the necessary preventative measures are not taken. Therefore new strategies are needed to effectively manage these diseases, which include addressing both modifiable and non-modifiable risk factors for patients with NCDs.

The aim of the current study was to develop and validate a pharmacogenomics profiling panel suitable for the individualizing metformin therapy for patients from the Bantu populations in South Africa. The individualization of metformin therapy has the potential to reduce the incidence of uncontrolled T2DM among patients taking this first-line anti-diabetic drug.

The main objective of part 1 of the study (**Chapter 2**) was to explore the prevalence and risk factors of DM and HTN in South Africa, especially within the economically disadvantaged population. More importantly, the extent of uncontrolled DM and HTN among resource-constrained patients receiving

treatment in Cecilia Makiwane hospital, serving the rural areas in Mdantsane. In part 2 of the study (**Chapter 3**), nineteen pharmacogenomics biomarkers were evaluated for their suitability for individualized metformin therapy for T2DM Patients. A genetic association study was conducted to investigate the level of association between nineteen pharmacogenomics biomarkers (SNPs) and response to metformin treatment, and to evaluate their suitability for individualizing metformin therapy for diabetic patients from the Bantu populations.

Chapter Two

Socio-demographic and modifiable risk factors of diabetes and hypertension among resource constrained patients from rural areas in Mdantsane Township in South Africa

2.1. Abstract

Introduction: Diabetes mellitus and hypertension have been identified as the leading causes for the rise in non-communicable diseases worldwide. The four major risk factors contributing to the non-communicable diseases burden are: tobacco use, physical inactivity, unhealthy diets and alcohol consumption. Insight into the effects that risk factors have on non-communicable diseases such as diabetes mellitus and hypertension is crucial for effective management and treatment of these diseases in under-studied populations. ***Aim:*** To demonstrate the socio-demographic and modifiable risk factors of diabetes mellitus and hypertension among South Africans adult residing in resource-constraint Mdantsane Township. ***Methods:*** A cross-sectional analytical study was conducted in the Cecilia Makiwane Hospital serving the residents of Mdantsane from July 2017 – October 2017. Relevant data on socio-demographic, anthropometric measurements, triplicate blood pressure , fasting blood glucose and lipogram analysis were obtained from 265 outpatients (18 years and older). ***Results:*** Multivariate analysis shows no salt intake, never smoke, normal triglyceride and normal high-density lipoprotein levels were significantly associated with reduced risk of DM with adjusted odds ratio of 0.21 (95% CI: 0.08-0.61; p=0.004), 0.12

(95% CI:0.03-0.43; *p*=0.001), 1.23 (95% CI: 0.73-2.06; *p*=0.013) and 0.16 (95% CI: 0.21-1.29; *p*=0.003), respectively. Underweight and normal-weight were significantly associated with a lower risk of hypertension with odds ratio of 7.98 (95% CI: 2.02- 31.53; *p*=0.003) and 19.17 (95% CI: 2.53-145.20; *p*=0.004), respectively. ***Conclusion:*** The impact of diabetes mellitus and hypertension to society can be drastically reduced with simple lifestyle changes. Thus, preventative strategies need to be addressed for large-scale screening and better management of these diseases to reduce the burden of Diabetes mellitus and hypertension in South Africa and worldwide.

2.2. Introduction

Non-communicable diseases (NCDs) have currently been identified as the leading cause of death worldwide. In the past decade developing countries have shown a dramatic increase in NCDs (Van de Vijver *et al*, 2014; WHO, 2016). The burden of NCDs in South Africa has increased over the past years resulting in an estimated 37% of all-cause mortality and 16% of disability-adjusted life years (Alberts *et al*, 2005; Puoane *et al*, 2012; Maimela *et al*, 2016). Currently, diabetes mellitus (DM) and hypertension (HTN) are the two most prevalent NCDs associated with the rapid increase in mortality (WHO, 2015a; Kearney *et al*, 2005; Opie and Seedat, 2005, Centers for disease control and prevention, 2012; WHO, 2012).

DM is defined as a chronic health condition associated with elevated blood sugar levels (American diabetes association, 2014; Pradeep and Haranath, 2014), whilst HTN is characterized by a systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg (WHO, 2016). DM often co-exists with HTN since they both share common disease mechanisms and in some instances, the one condition exacerbates the other (Dokumnu *et al*, 2018). Currently, 425 million people are diagnosed with diabetes, whilst it is estimated that over a billion people worldwide are affected with HTN (WHO, 2016; IDF, 2016). Both diseases have strongly been associated with an increased risk of kidney failure, obesity, stroke, blindness, nerve damage and cardiovascular diseases (CVD) (WHO, 2016; Todd and Florez, 2014; Williams, 1994; WHO, 2003; 2015c).

DM and HTN have been shown to have a major impact on public health funding consuming a significant proportion of public health spending (Kearney *et al*, 2005) However, these are described as lifestyle diseases, thus they can be prevented or managed by drugs and lifestyle modification (Diabetes Prevention Program Research Group, 2002; Griffin *et al*, 2011; Herman *et al*, 2015). Modifiable risk factors associated with DM and HTN include tobacco use, alcohol consumption, physical activity and unhealthy diets (Forouzanfar *et al*, 2016). Low- and middle-income countries are the most affected by these risk factors (Adeniyi *et al*, 2016; WHO, 2010).

In South Africa, the burden of NCDs is predicted to increase substantially in the next decades if the necessary preventative measures are not taken (Mayosi *et al*, 2009). Furthermore, strategies need to be implemented to effectively manage these diseases (Ntuli *et al*, 2015). Currently, there are limited studies exploring the prevalence and risk factors of DM and HTN in South Africa, especially within the economically disadvantaged population. To address the aforementioned problem, the current study was initiated to evaluate the socio-demographic and modifiable risk factors of DM and HTN in Cecilia Makiwane hospital, serving the rural areas in Mdantsane. This study generates local influential factors that contribute to the development of these diseases. Modifiable risk factors found significantly associated with diabetes and/or hypertension will be used to promote health education as primary prevention.

2.3. Materials and Methods

2.3.1. Study area and design

A cross-sectional analytical study was conducted in the Cecilia Makiwane Hospital (Mdantsane, South Africa) from July 2017 – October 2017. Mdantsane is located in the Buffalo Municipality and is a low-income residential township with a population of approximately 150000 (Census, 2011). The objectives of the study were explained to all participants and each participant signed a consent form indicating voluntary participation in the study. Information sheets were provided in both English and IsiXhosa languages. Prior to sampling, participants underwent a physical examination and medical history data were recorded.

2.3.2. Study population and sampling

Inclusion criteria for participants in this study were individuals aged ≥18 years and have been diagnosed with hypertension and/or diabetes for more than a year prior to the study. Exclusion criteria included pregnant women, patients diagnosed with type 1 diabetes and acute illnesses. Age, gender, monthly income, level of education, lifestyle profile (i.e. physical activity and diet), family history of disease prevalence, smoking status and alcohol status were obtained through the interview from all of the participants. The use of anti-hypertensive and antidiabetic medications along with the duration of disease(s) was obtained from the patients' medical records. Eligible participants (N=265) were recruited sequentially at the study setting over the study period.

2.3.3. Data collection

A trained research nurse conducted anthropometric measurements of weight to the nearest 0.1 kg, height to the nearest of 0.1 cm using a stadiometer, waist circumference, hip circumference, and upper-arm circumference was measured using a tape measure. Measurements were taken with all participants wearing minimal clothing and no shoes. Blood pressure (BP) was measured using a validated automated digital blood pressure monitor (Microlife® BP A100 Plus). BP was recorded in triplicate and the average was used for analysis at each visit. Blood glucose was measured using Accutrend ® test strips. Body mass index (BMI) for each patient was calculated as weight (kg) divided by height (m^2) and was categorized based on WHO criteria (year): underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/ m^2), overweight (25.0-29.9 kg/ m^2) and obese (30 or greater kg/ m^2). Patients with systolic BP (SBP) of ≥ 140 mmHg and ≥ 90 diastolic BP (DBP) were identified as hypertensive and patients with systolic and diastolic BP below 140 mmHg and 90 mmHg respectively were identified as normotensive.

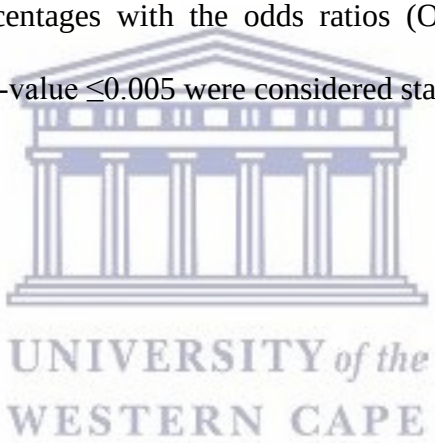
2.3.4. Laboratory assessment

Fasting venous blood was obtained for all patients. The lipid profile [which includes: total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL)] for each participant was categorized according to the guidelines of the Heart and Stroke Foundation of South Africa (Maimela *et al*, 2016; The Heart and Stroke Foundation, 2017). In addition to this, the glycosylated hemoglobin (HbA1c) was assayed from blood samples of diabetic participants (Amod, 2012). All blood samples were sent to the clinical laboratory

center, i.e. National Health Laboratory Services (NHLS) of Cecilia Makiwane hospital and the East London private hospital.

2.3.5. Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 25 for Windows (SPSS Inc., Chicago, IL, USA). The clinical laboratory data and anthropometric measurements were expressed as mean (n) ± standard deviation (SD). Differences between groups were assessed using a chi-square test for statistical significance. Risk factors associated with DM and HTN are presented as percentages with the odds ratios (ORs) and 95% confidence intervals (CIs). The p-value ≤ 0.005 were considered statistically significant.



2.4. Results

In the study cohort, a total of 265 outpatients (of which n=175 were female and n=90 were male) were interviewed during a 3-month study (**Table 2.1**). The mean ages of men and women were 59.96±11.19 and 61.32±11.60 years, respectively. Other demographic, anthropometric and clinical laboratory measurements of the study participants are indicated in (**Table 2.1**).

Table 2.1. Characteristics of the study subjects (n=265).

Parameter	Female (n=175)	Male (n=90)	Total (n=265)
Age (years)	59.96±11.19	61.32±11.6	60.42±11.32
Weight (Kg)	87.45±21.46	81.62±16.06	85.46±19.94
Height (cm)	159.93±6.22	168.10±11.66	162.72±9.30
BMI (Kg/m^2)	34.18±8.27	29.77±12.93	32.68±10.29
HbA1c (%)*	10.40±2.80 (n=85)	10.48±3.91 (n=32)	10.42±3.12 (n=117)
FBG (mmol/l)*	12.65±5.11 (n=85)	13.11±3.92 (n=32)	12.78±4.80 (n=117)
Systolic (mmHg)	155.67±20.53	157.04±21.65	156.14±20.88
Diastolic (mmHg)	92.56±13.18	93.87±13.45	93.00±13.26
Heart rate (pbm)	84.14±13.60	80.01±14.43	82.74±13.99
TC (mmol/L)	5.02±1.27	4.53±1.14	4.86±1.25
HDL (mmol/L)	1.30±0.36	1.35±0.44	1.31±0.39
LDL (mmol/L)	2.63±1.14	2.35±0.98	2.54±1.09
TG (mmo/L)	1.71±1.01	1.88±1.06	1.77±1.03

BMI – Body Mass Index, HbA1c - glycated hemoglobin, FBG-Fasting blood glucose, TC – Total Cholesterol, HDL – high-density lipoproteins, LDL – Low-Density Lipoproteins, TG – Triglycerides, CRT – Creatinine, GFR - glomerular filtration rate. n – Total number of samples/patients, P-value > 0.05. * HbA1c and RBG were only measured for patients diagnosed with DM thus n vary. Values are presented as means ± standard +error of the mean

Table 2.2 and **2.3** indicates the socio-demographic and modifiable risk factors of non-diabetic and diabetic groups as well as among non-hypertensive and hypertensive groups. It is important to note that approximately 40% of the study cohort was co-morbid. In both NCDs, the proportion of females is higher than males, however, gender was only shown to be significantly associated amongst diabetic patients (*p*-value = 0.043). Amongst diabetic patients, smoking status; salt intake, TG and HDL were all significantly associated with disease incidence with *p*-values of 0.015; 0.004, 0.012 and 0.003 respectively (**Table 2.2**). All other factors, i.e. age, educational level, physical activity, alcohol consumption, TC and LDL were not significantly associated with DM (**Table 2.2**). BMI was the only modifiable risk factor that showed significant association amongst hypertensive patients with a *p*-value of <0.0001 (**Table 2.3**). Factors not significantly associated with HTN were: gender, age, educational level, smoking status, physical activity, salt intake, and alcohol consumption, TC, TG, LDL and HDL (**Table 2.3**).

Table 2.2. Socio-demographics and Modifiable risk factors among diabetes (n=265).

Variables	Subgroups	Group				X^2, *P*-Value
		Non-diabetic		Diabetic		
		n = 148	%	n=117	%	
Gender	Male	58	39.2	32	27.4	
	Female	90	60.8	85	72.6	**4.08, 0.040**
Age	Less than 50 years	28	18.9	16	13.7	
	More than 50 years	120	81.1	101	86.3	1.30, 0.260
Educational level	Uneducated	12	8.2	8	6.8	
	Primary	34	23.1	29	24.8	4.78, 0.190
	Secondary	97	66.0	70	59.8	
	High education	4	2.7	10	8.5	
Smoking status	Never smokers	99	66.9	91	77.8	
	Quit smokers	29	19.6	22	18.8	**8.45, 0.015**
	Current smokers	20	13.5	4	3.4	
Physical activity	More than 3 times / week	8	5.4	8	6.8	0.25, 0.880
	1-2 times/ week	119		92		
	No physical activities	21	80.4	17	78.6	
Salt intake	No salt intake	11	14.2	18	14.5	
	Normal salt intake	103	7.4	88	15.4	**11.15, 0.004**
	Increased salt intake	34	69.6	11	75.2	
Alcohol consumption	Never drank	78	23.0	64	9.4	3.63, 0.160
	Quit drinking	40	52.7	39	54.7	
	Occasional drinker	30	27.0	14	33.3	
BMI (Kg/m^2)	<18.5	4	20.3	3	12.0	
	18.5-24.9	21	2.7	17	2.6	
	25.0-29.9	37	14.2	32	14.7	0.28, 0.960
	≥30	86	25.0	64	27.6	
TC (mmol/L)	Increased	52	58.1	59	55.2	
	Normal	96	35.1	58	50.4	0.15, 0.700
TG (mmol/L)	Increased	52	64.9	59	49.6	
	Normal	95	35.4	58	50.4	**6.28, 0.012**
HDL (mmol/L)	Decreased	74	64.6	36	49.6	
	Normal	69	51.7	73	33.0	**8.81, 0.003**
LDL (mmol/L)	Increased	50	48.3	38	67.0	
	Normal	98	33.8	79	32.5	0.05, 0.820

TC= Total Cholesterol, TG= Triglyceride, HDL= High density lipoprotein, LDL= Low density lipoprotein, mmol= mill mole, L= litre

Table 2.3: Factors affecting the modifiable risk factors of hypertension in study subjects (n=265).

Variables	Subgroups	Group				X^2, *P*-value
		Non-hypertensive		Hypertensive		
		n = 13	%	n= 252	%	
Gender	Male	6	46.2	84	33.3	0.91, 0.34
	Female	7	53.8	168	66.7	
Age	Less than 50 years	4	30.8	40	15.9	1.98, 0.16
	More than 50 years	9	69.2	212	84.1	
Educational level	Uneducated	0	0	20	8.0	1.46, 0.69
	Primary	4	30.8	59	23.5	
	Secondary	8	61.5	159	63.3	
	High education	1	7.7	13	5.2	
Smoking status	Never smokers	8	61.5	182	72.2	1.17, 0.56
	Quit smokers	4	30.8	47	18.7	
	Current smokers	1	7.7	23	9.1	
Physical activity	More than 3 times / week	0	0	16	6.3	0.88, 0.64
	1-2 times/ week					
	No physical activities	11	84.6	200	79.4	
Salt intake	No salt intake	2	15.4	36	14.3	1.84, 0.40
	Normal salt intake	0	0	29	11.5	
	Increased salt intake	10	76.9	181	71.8	
Alcohol consumption	Never drank	3	23.1	42	16.7	3.32, 0.19
	Quit drinking	4	30.8	138	54.8	
	Occasional drinker	5	38.4	74	29.4	
BMI (Kg/m^2)	<18.5	4	30.8	40	15.9	**21.34, <0.0001**
	18.5-24.9	2	15.4	5	2.0	
	25.0-29.9	6	46.2	32	12.7	
	≥30	1	7.7	68	27.1	
TC (mmol/L)	Increased	4	30.8	146	58.2	0.001, 0.98
	Normal	5	38.4	98	38.9	
TG (mmol/L)	Increased	8	61.5	154	61.1	0.69, 0.41
	Normal	4	30.8	107	42.5	
HDL (mmol/L)	Decreased	9	69.2	145	57.5	2.37, 0.12
	Normal	2	15.4	108	44.6	
LDL (mmol/L)	Increased	8	61.5	134	55.4	0.04, 0.85
	Normal	4	30.8	84	33.3	

TC= Total Cholesterol, TG= Triglyceride, HDL= High density lipoprotein, LDL= Low density lipoprotein, mmol= mill mole, L= litre, statistically significant are in bold (*p*-value-0.05)

Table 2.4 and **2.5** represents results of univariate and multivariate analysis for diabetes and hypertension. The univariate results show an association between diabetes and smoking status, salt intake, TG and HDL (**Table 2.4**). The results gave an indication that smoking status has an impact on diabetes using current smokers as the baseline, those who have quit smoking had significantly lesser odds (*p*-value = 0.055) of diabetes, while the odds of those who never smoked was even further reduced (*p*- value = 0.001) (**Table 2.4**). Furthermore, no salt intake, normal TG and normal HDL-C levels demonstrated significantly higher odds (*p*-value < 0.01) in decreasing the risk of diabetes. Gender was not significantly associated with diabetes (*p*-value = 0.210). In **table 2.5**, participants who are overweight (as per WHO standards) have a higher risk of hypertension (*p*-value = 0.580). A decreased in BMI in comparison to obese has higher odds in decreasing the risk of hypertension (*p*-value=0.004 and *p*-value=0.006).

Multivariate logistic regression analysis showed that after adjusting for all significant factors, "increased salt intake" and "never smoked" were significantly associated with DM and BMI was significantly associated with HTN **(Table 2.4** and **2.5).**

Table 2.4. Univariate and multivariate analysis for risk factors of diabetes status

Factors	Diabetic N (%)	Non-Diabetic N (%)	Unadjusted Odds ratio	Adjusted odds ratio	*P*-value
Gender					
Male	58 (39.2)	32 (27.4)	1	1	
Female	90 (60.8)	85 (72.6)	1.71 (1.01-2.89)*	1.55 (0.78-3.1)	0.212
Salt intake					
Increased salt intake	34 (30.0)	11 (9.4)	1	1	
Normal salt intake	103 (69.6)	88 (75.2)	2.64 (1.26-5.52)	0.55 (0.24-1.25)	0.153
No salt intake	11 (7.4)	18 (15.4)	5.06 (1.84-13.92)*	0.21 (0.08-0.61)*	0.002*
Smoking status					
Current smokers	20 (13.5)	4 (3.4)	1	1	
Quit smoking	29 (19.6)	22 (18.8)	0.83 (0.44-1.54)	0.27 (0.07-1.03)	0.055
Never smoke	99 (66.9)	91 (77.8)	0.26 (0.08-0.88)*	0.12 (0.03-0.43)*	0.031*
TG- Cholesterol					
Increased level	52 (35.1)	59 (50.4)	1	1	
Normal level	96 (64.9)	58 (49.6)	0.53 (0.33-0.87)*	1.23 (0.74-2.06)	0.013*
HDL-Cholesterol					
Decreased level	74 (51.7)	36 (33.0)	1	1	
Normal level	69 (48.3)	73 (67.0)	0.46 (0.27-0.77)*	0.16 (0.21-1.29)	0.003*

Statistically significant are in bold (*P-value <0.05)

Table 2.5. Univariate and multivariate analysis for risk factors of hypertension

Factors	Hypertensive N (%)	Non-hypertensive N (%)	Unadjusted Odds ratio	Adjusted odds ratio	P-value
BMI					
>30	4 (30.8)	146 (58.2)	1	1	
25.0-29.9	1 (7.7)	68 (27.1)	1.86 (0.20-17.0)	0.58 (0.06-5.40)	0.635
18.5-24.9	6 (46.2)	32 (12.7)	0.15 (0.04-0.55)*	7.98 (2.02-31.53)*	0.003*
<18.5	2 (15.3)	5 (2.0)	0.07 (0.01-0.47)*	19.17 (2.53-145.2)*	0.004*
Smoking status					
Current smokers	1 (4.2)	23 (9.1)	1	1	
Quit smoking	4 (7.8)	47 (18.7)	0.51 (0.05-4.83)	3.29 (0.31-34.89)	0.323
Never smoke	8 (4.2)	182 (72.2)	0.99 (0.12-8.27)	3.41 (0.34-34.70)	0.300
Alcohol consumption					
Occasional drinker	4 (30.8)	40 (15.9)	1	1	
Quit drinking	5 (38.4)	74 (29.3)	0.68 (0.17-2.66)	0.54 (0.12-2.38)	0.417
Never drank	4 (30.8)	138 (54.8)	0.29 (0.07-1.21)	0.38 (0.08-1.74)	0.212

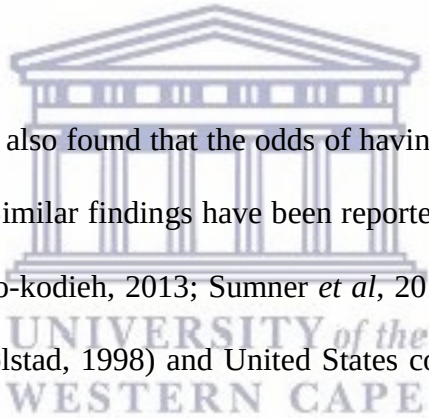
Statistically significant are in bold (*P-value <0.05)

2.5. Discussion

The African region has been identified to have the highest burden of DM and HTN (IDF, 2016; WHO, 2015b). In Africa, DM is estimated at 15.5 million adults aged between 20-79 years (IDF, 2017) and HTN is estimated at 46% in adults >25 years (WHO, 2015b). The ever-increasing numbers of individuals diagnosed with these diseases are of great concern across the world especially in middle- and low-income countries (WHO, 2015b). The present study highlights the burden and associated risk factors of DM and HTN in Mdantsane, a resource-constrained township of South Africa.

South Africa has been reported to have the highest incidence of DM in the African continent (IDF, 2016). Amongst the modifiable risk factors, a significant association with decreased risk was shown with no tobacco intake, no salt intake, normal level TG and HDL. Tobacco smoking is well established as a risk factor for multiple diseases and has been associated with DM in multiple cohort studies (US Department of Health and Human Services, 2004a; Willi *et al*, 2007; Cassano *et al*, 1992). The present study showed that smoking was associated with the probability of developing DM. This finding is consistent with previous studies conducted in Korea (Jee *et al*, 2003; Kim and Oh, 2013). Current smokers and ex-smokers display a greater probability of developing DM than non-smokers, however, in this study, the increased risk of ex-smokers were not statistically significant. Previous studies conducted by Jee *et al*. (2010) and Hur *et al*. (2007) also reported the increased risk of ex-smokers as insignificant (Jee *et al*, 2010; Hur *et al*, 2007).

WHO (2016) recommends that patients with DM should reduce their dietary salt intake (WHO, 2016). The precise relationship between dietary salt intake and DM is not well defined; however, excessive salt intake is well associated with hypertension and CVDs. In the present study, increased salt intake was significantly associated with the incidence of DM. Previous studies also demonstrated an association between high dietary salt intake and DM (Hu *et al*, 2005; Ekinci *et al*, 2010; 2011). Increased TG levels have been associated with an increased risk of DM (Experience of an international collaborative group, 1982; Nomura *et al*, 1991; Sorlie and Feileib, 1982) and in this study cohort, similar results were observed.



In addition, this study also found that the odds of having DM were increased with a decrease in HDL. Similar findings have been reported in African (Motala *et al*, 2011; Tagoe and Amo-kodieh, 2013; Sumner *et al*, 2010; Fagot-Campagna *et al*, 1999), European (Njølstad, 1998) and United States communities (Haffner *et al*, 1990; Montonen *et al*, 2011). Lower levels of HDL concentrations have been associated with many diseases such as CVDs (Gordon *et al*, 1977, Gordon *et al*, 1989; Sharrett *et al*, 2001; Karadag *et al*, 2009), nephropathy (Morton *et al*, 2012) and coronary heart disease (Kucharska-Newton, 2008; Filippatos and Elisaf, 2013). Although levels of TC and LDL in diabetic individuals are reportedly comparable with that found in non-diabetics, low levels of HDL and elevated TG have been reported in T2DM patients as the probable cause of CVD (Gordon *et al*, 1977; 1989; Sharrett *et al*, 2001). It has also been observed that HDL alone might not be a good indicator of increased DM risk since most of the subjects had lower total

cholesterol. Moreover, lower levels of HDL in the present study might be because of the lower cholesterol.

A high BMI is a risk factor that is often associated with DM (Al-Nsour, 2012), however, in this study; it was significantly associated with HTN since DM and HTN co-exist in approximately 40% of the study cohort, this could be an explanation for this observation. Furthermore, many studies suggest that a high BMI contributes to hypertension (Vasan *et al*, 2002; Redon *et al*, 2008; Rudetsikira *et al*, 2012; Mulenga *et al*, 2013; Reddy and Prabhu, 2005). It is well established that smoking increases the risk of hypertension; however, the significance of this association may differ between populations (Dhungana *et al*, 2016). In this study, no significant association was observed between hypertension and smoking status. These findings are contrary to other studies (Pandey *et al*, 2009; Shanthirani *et al*, 2003; Singh *et al*, 2011, Mohan *et al*, 2007).

2.6. Summary

It can be concluded that there is a significant burden of DM and HTN in the rural areas of Mdantsane, Eastern Cape. In summary, a lower risk of DM was associated with no "salt intake", "never smoke", and normal levels of TG and HDL whilst a lower risk of hypertension was associated with decreased BMI. Development of best practices for affordable and effective programs in screening, prevention, detection and treatment of DM and HTN is essential. In order to reduce the burden of NCDs, comprehensive intervention strategies should be implemented across the country. Future studies with a larger sample size should be done to identify or generate local modifiable risk factors for the development of DM and HTN.

Chapter Three

Evaluation of the Suitability of Nineteen Pharmacogenomics Biomarkers for Individualized Metformin Therapy for Type 2 Diabetes Patients

3.1. Abstract

Introduction: Type 2 diabetes mellitus is a progressive metabolic disease characterized by relative insulin insufficiency and insulin resistance resulting in hyperglycemia. Metformin is currently a first-line drug used to treat type 2 diabetes. Despite its widespread use, there is considerable variation in response to metformin; with more than one-third of the patients failing to achieve adequate glycaemic control. Numerous studies have reported the involvement of single nucleotide polymorphisms and their interactions in genetic pathways i.e. pharmacodynamics and pharmacokinetics of the drug. ***Aim:*** to investigate the genetic association between nineteen pharmacogenomics biomarkers (SNPs) and response to metformin treatment, and to evaluate their suitability for individualizing metformin therapy for diabetic patients. ***Methods:*** Two multiplex MassARRAY systems (Agena Bioscience™) were designed and optimized by Inqaba Biotechnical Industries (Pretoria, South Africa), and used for the genotyping of the selected SNPs for 140 T2DM outpatients. ***Results:*** The CT genotype of the *FMO2* rs12752688 polymorphism was significantly associated with increased response to metformin therapy (OR= 0.33, 95% CI [0.16-0.68], *p*-value= 0.003). A moderate association was also found between the GA genotype of *SLC47A2* rs12943590 and

a decreased response to metformin therapy (OR= 2.29, 95% CI [1.01-5.21], *p*-value=0.048 for the heterozygous GA genotype. ***Conclusion:*** To our knowledge, this is the first study that investigated the association between genetic variants and responsiveness to medication for diabetic patients from the indigenous Bantu-population of South Africa. The *FMO2* rs12752688 polymorphism is suggested to be included in pharmacogenomics profiling systems developed to individualize metformin therapy for diabetic patients from the Bantu populations.

3.2. Introduction

The prevalence of diabetes mellitus (DM) is increasing at an alarming rate and imposing a burden on the economy worldwide (Pheiffer *et al*, 2018; WHO, 2018b). To date, diabetes remains the leading cause of premature deaths and cardiovascular diseases globally (IDF, 2017; Ogurtsova, 2017). Currently, 415 million people in the world are living with diabetes and the number is expected to increase to 629 million by the year 2045, and about 55% of these cases are expected to be of type 2 diabetes mellitus (T2DM) (IDF, 2017). According to the International Diabetes Federation (IDF), 15.5 million adults in Sub-Saharan Africa between the ages of 20 and 79 are living with diabetes, with the highest prevalence observed amongst adults between the ages of 55 and 65. Moreover, within the Southern African region, there is a high prevalence of T2DM and has increased significantly in the last decade, particularly in urban dwelling populations (IDF, 2017).

T2DM, also referred to as insulin-independent diabetes, is a progressive metabolic disease that is characterized by obesity, impaired insulin action, insulin secretory dysfunction and increased endogenous glucose output (White, 2003; Donath *et al*, 2005; Nieto-Vazquez *et al*, 2008; Tara *et al*, 2008; Abdul-Ghani and DeFronzo, 2010; Babu *et* al, 2013). Diet and other lifestyle modifications are usually the initially implemented therapies in T2DM management (Inzucchi *et al*, 2012). However, the use of oral anti-diabetic (OAD) drugs plays a key role in disease progression and management (Sharifali *et al*, 2010; Daniels *et al*, 2016). The commercially available

OADs include biguanides such as metformin, sulfonylureas (SUs), thiazolidinediones (TZDs), meglitinides, insulin and glucose-like peptides. Amongst these drugs, metformin remains the most commonly prescribed anti-diabetic drug worldwide (Daniels *et al*, 2016; Inzucchi *et al*, 2015). Despite metformin's widespread use, its mechanism of action is not fully understood. Even so, studies have shown that it alleviates hyperglycemia without imposing the risk of hypoglycemia (Hundal *et al*, 2000, Johnson *et al*, 2002; Bruijstens *et al*, 2008; Cho *et al*, 2015; Roumie *et al*, 2016). Metformin exerts its anti-diabetic properties through inhibiting the production of hepatic glucose, reducing intestinal glucose absorption, and improving glucose uptake and utilization in peripheral tissue (Hundal *et* al, 2000; Cho *et al*, 2015; Argaud *et al*, 1993). Moreover, the drug is also known to improve, oxidative stress, insulin resistance, endothelial dysfunction and fat distribution in T2DM patients (Alexandre *et al*, 2008; Wang *et al*, 2013; Kristensen *et al*, 2014; Duca *et al*, 2015; Chen *et al*, 2015b).

The estimated percentage of adherence in T2DM patients ranges from 36% - 93% on 6-24 months treatment (Cramer and Pugh, 2005; García-Pérez *et al*, 2013). Thus far, about one-third of patients fail to obtain acceptable glycaemic control with metformin monotherapy (Fonseca, 2009; Diabetes Prevention Program Research Group, 2012; Desai *et al*, 2012). Literature suggests that inter-individual variability in the efficacy together with adverse drug reactions are common amongst patients undergoing metformin therapy. These differences are brought by a variety of factors such as

gender, age, body weight, lifestyle and most likely genetic factors. Genetic variations in drug metabolizing enzymes, as well as drug transporters, have been shown to account for the differences in drug response among patients (Evans and Johnson, 2001). Furthermore, genetic variation presented by drug transporter genes such as organic cation transporters (OCTs) and multidrug and toxin extrusion (MATEs) which are involved in metformin transport, have been shown to directly influence the pharmacodynamics as well as the pharmacokinetics of the drug Becker *et al*, 2009b; He *et al*, 2015; Shokri *et al*, 2016).

Although some variants presented in the form of SNPs have been accounted for, there is still a large number that has not been associated with the T2DM phenotype or metformin use, particularly in populations of African origin. The African population presents a great genetic variation in comparison to non-African populations (Krause, 2015). The South Africa population presents diverse genetic variability, which has offered unique opportunities to researchers in the field of human and medical genetics (Krause, 2015; Jacobs *et al*, 2014). The genetic diversity presented by the indigenous populations of South Africa is relevant for improving diagnostic techniques as well as the efficacy of treatment for complex medical conditions such as DM, cardiovascular diseases and cancer. Moreover, studying the genetic variation within these populations is relevant in the context of precision medicine, which aims to customize healthcare, with medical decisions, treatments, practices, or products being tailored to the individual patient. Therefore, the aim of this study was to investigate the genetic association between nineteen pharmacogenomics biomarkers (SNPs) and response to

metformin treatment and to evaluate their suitability for individualizing metformin therapy for diabetic patients from the indigenous Bantu population of South Africa.

3.3. Material and Methods

3.3.1. Patients and study design

All participants were briefed about the project and a consent form was completed and submitted by each participant before the experiment was conducted. Ethical clearance for this study was obtained from the Senate Research Committee of the University of the Western Cape (Ethics approval number BM/16/5/19)

3.3.2. Patient selection

A total of 140 T2DM outpatients belonging to the indigenous Bantu (Swati, Xhosa and Zulu) population groups of South Africa were recruited from Cecilia Makiwane Hospital (East London, Eastern Cape) and Piet Retief Hospital (Mkhondo, Mpumalanga). Patients recruited were 18 years or older, and were on continuous metformin treatment for at least 6 months. Pregnant patients, as well as those who were undergoing treatment for conditions such as Type 1 diabetes mellitus (T1DM), malignancies, chronic kidney and liver diseases, were excluded from the study. Demographic information, socioeconomic profile, family history, as well as the dietary background were obtained using questionnaires. Clinical data such as anthropometric measurements, blood pressure, serum glycosylated hemoglobin (HbA1c) and prescription of drugs, was obtained from each patient's medical file.

3.3.3. Sample collection

Fasting venous blood was collected to measure: HbA1c levels and lipid profiles. Genomic samples were collected in the form of buccal swabs and frozen at -20 °C until further use. For each patient, HbA1c was measured within 6-month (baseline) and 12-month (follow up) periods. Based on the HbA1c levels, patients (taking up to 2550 mg of metformin per day) were divided, according the classification used by Kashki *et al*. (2015), CDE (2018) and Amod (2017), to: (1) responder group (decrease in HbA1c values less than 8% at 12 months compared to baseline prior to the study) and (2) non-responder group (increase in HbA1c values more than 8% at 12 months compared to baseline prior to the study). HbA1c averages of the responders and non-responders are shown in **Figure 3.1.**

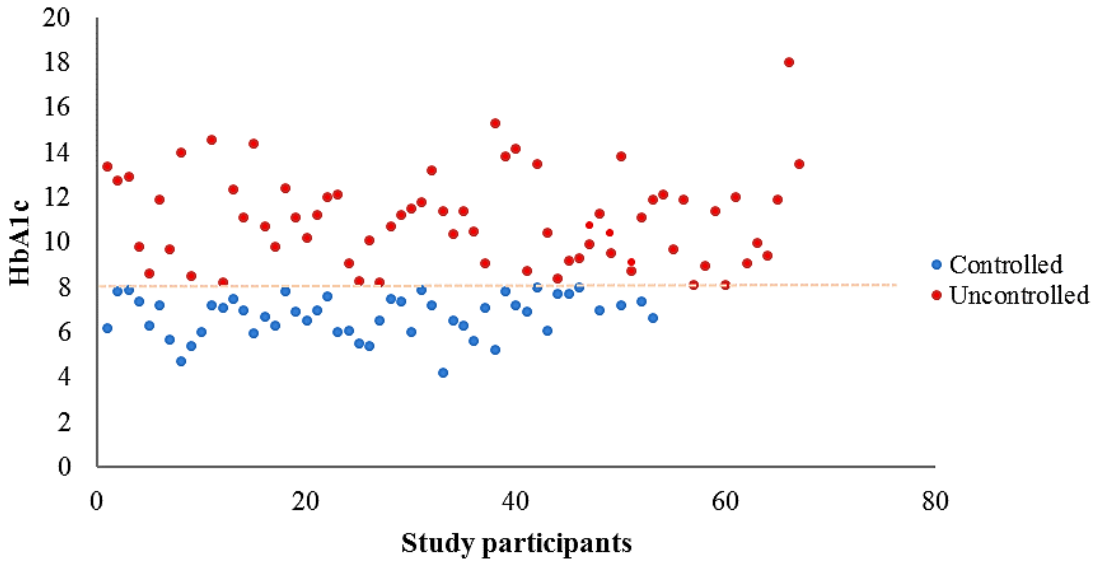


Figure 3.1. A scatter plot illustration of HbA1c categories for T2DM patients. An HbA1c percentages category versus responders and the non-responder patient's on metformin therapy.

3.3.4. DNA isolation

Genomic DNA was extracted from buccal swabs samples using a standard salt-lysis procedure (Lahiri, 1991). Samples were stored at -20 °C until further use. DNA quantification was conducted using a NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific™) and Gel Doc™ EZ Gel Documentation System (BIO-RAD, USA).

3.3.5. Selection of pharmacogenomics biomarkers

Nineteen Single nucleotide polymorphisms associated with response to metformin treatment for T2DM were selected using the Pharmacogenomics knowledge base (www.pharmgkb.org), Ensembl (http://www.Ensembl.org), as well as an extensive survey of recent literature.

3.3.6. Genotyping

Two multiplex MassARRAY systems (Agena Bioscience™) were designed and optimized by Inqaba Biotechnical Industries (Pretoria, South Africa), and used for the genotyping of the selected SNPs for 140 T2DM outpatients. **Figure 3.2** shows an example of one SNP variant which was genotyped using MassARRAY®System IPLEX extension reaction.

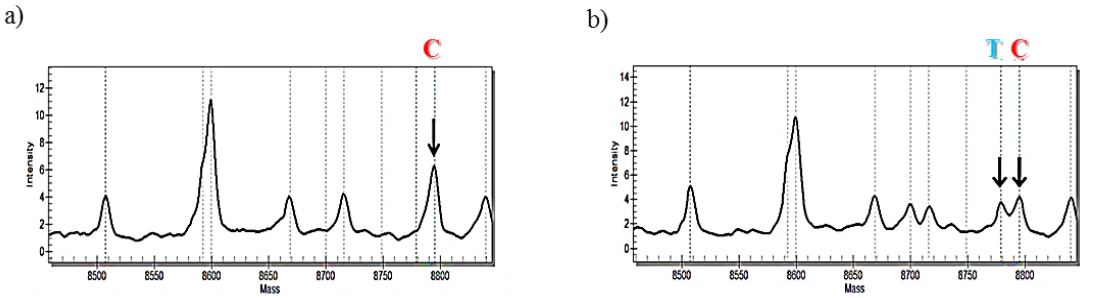


Figure 3.2. Examples of genotype call for *FMO2*: rs12752688 by the electropherogram of MassARRAY®. a).The genotype call for a homozygous wild-type sample for *FMO2*: rs12752688. b) The genotype call for a heterozygous sample for *FMO2*: rs12752688. The arrows indicate the nucleotide(s) of interest.

3.3.7. Statistical analyses

Statistical analyses were performed using Microsoft Excel version 10.0.40820 and Medcalc version 2.2.0.0. Data were expressed as mean (n) ± standard deviation (SD) and percentages (%) for continuous and categories variables respectively. Minor allele frequency (MAF) and Hardy-Weinberg equilibrium (HWE) test were calculated for all the SNPs. Associations between alleles and genotypes and response to metformin were measured using odds ratios (ORs), 95% confidence interval (95%CI) and *p*-value derived from unconditional logistic regression. *P*-value <0.05 was considered significant.

3.4. Results

3.4.1. Clinical and laboratory characteristics of study participants

This study included 106 (76%) women and 34 (24%) men patients with a mean age of 59.9 ± 11.2 years. The patients were split into two groups: controlled (n=53) and uncontrolled (n=87). The clinical and biochemical characteristics of the controlled and uncontrolled study groups on metformin therapy are shown in **Table 3.1**.

Table 3.1. Clinical and biochemical characteristics of the study participants with controlled and uncontrolled metformin response

Parameter		**Controlled(n=53)**	**Uncontrolled (n=87)**	***p*-value**
Gender (F/M)		36/14	57/30	-
Ethnicity	**Xhosa[1]**	18	63	-
	Zulu[2]	21	28	-
	Swati[2]	6	4	-
Age		60.7 ±11.0	58.3 ±11.4	0.470
Weight (Kg)		85.8 ± 19.5	85.4 ± 19.1	0.833
Height (cm)		162.1 ± 7.8	163.1 ± 7.7	0.479
BMI		31.9 ± 8.4	30.4 ± 10.7	0.304
HbA1c	**6 months**	7.6 ± 2.0	11.0 ± 2.0	<0.001
	12 months	6.7 ± 1.2	11.5 ± 2.9	<0.001
RBG		9.4 ± 3.9	14.5 ± 6.6	<0.001
Systolic mmHg		147.0 ± 24.0	153.5 ± 23.9	0.130
Diastolic mmHg		83.9 ± 15.5	90.3 ± 13.7	0.018
TC (mmol/L)		4.4 ± 1.1	5.0 ± 1.1	0.001
HDL (mmol/L)		1.2 ± 0.4	1.2 ± 0.4	0.672
LDL (mmol/L)		2.3 ± 0.9	2.8 ± 0.9	0.007

TG (mmo/L)	2.0 ± 1.2	2.3 ± 1.1	0.215
Creatinine	157.7 ± 383.1	79.3 ± 29.5	0.317
GFR	41.7 ± 21.5	52.6 ± 25.0	0.313

Kg – Kilograms, cm – centimeter, BMI - Body Mass Index, HbA1c – Glycated Haemoglobin levels, RBG –Random blood sugar, TC Total Cholesterol, HDL – High-density lipoprotein, LDL – Low-density lipoprotein, TG –Triglycerides, CRT - Creatinine and GFR - Glomerular filtration rate. Values are presented as means ± standard error of the mean.1. Cecilia Makiwane Hospital, 2. Piet Retief Hospital

3.4.2. Comparison of minor allele frequency of nineteen SNPs in the Bantu to other world populations

The MAF observed for the selected SNPs were compared to world populations i.e. Luhya, Yoruba, African American, Japanese, British and Chinese Dai. SNPs rs784888 and rs784892 were mostly observed in African populations as compared to other ethnic non-African groups (**Table 3.2)**. Variant rs8187725 was not observed in the Bantu population either in the other chosen subpopulations (**Table 3.2)**. The allelic frequencies of the investigated SNPs were in accordance with HWE (p>0.05) except for rs2617102 (p=0.002), rs1920145 (p=0.043) and rs2076828 (p=0.030) (**Table 3.3)**. Moreover, only p-values below 0.00263157 (0.05/19) would be considered significant after a Bonferroni correction application (Butler, 2005). Therefore, all the SNPs are in accordance with HWE by showing no deviation from HWE except for rs2617102. The HWE analysis suggests that the study population does not change from one generation to the next and does not occur by chance alone (Hardy, 1908; Lee *et al*, 2008; Dorak, 2014; Butler, 2005; Nei & Kumar, 2000).

Table 3.2. Comparison of minor allele frequencies (MAF) of the nineteen selected SNPs of the Bantu population to other ethnic groups.

Gene	dbSNP	Variant	M > m	MAF						
				Bantu	Luhya*	Yoruba*	Africa American*	Japanese*	British*	Chinese Dai*
PPARGC1A	4:23864716	rs10213440 intronic	T > C	21.7	20.7	28.2	19.7	19.7	18.1	24.2
ATM C11orf65	11:108412434	rs11212617 intronic	C>A	17.5	18.7	18.1	36.9	40.4	59.3	39.2
SLC22A1	6:160122116	rs12208357 missense	C>T	0.4	0	0	1.6	0	6.0	0
SLC47A2	17:19716685	rs12943590 5 UTR	G>A	16.2	18.7	16.2	22.1	38.0	26.6	44.1
CSMD1	8:4606687	rs2617102 intronic	A>C	12.3	15.7	19.9	10.7	8.2	13.7	12.4
SLC22A1	6:160134722	rs594709 intronic	A>G	27.3	28.8	25.5	32.0	19.2	39.0	34.9
AMHR2	12.53429100	rs784892 intronic	G>A	35.6	33.8	37.0	17.2	0	0	0
SP1	12:53430724	rs784888 intronic	G>C	41.6	39.4	43.5	20.5	0	0	0
SLC47A2	17:19681658	rs34399035 Missense	C > T	0	0	0	0	0	0.5	0
SLC22A1	6:160136611	rs2282143 missense	C>T	7.2	6.6	9.3	4.1	15.4	2.2	12.4
FMO3	1:171076659	rs1920145 intergenic variant	T>C	53.6	46.0	51.9	41.0	54.8	35.2	50.0

Table 3.2. Continued

FMO2	1:171182499	rs12752688 intergenic variant	C > T	20.1	20.2	17.1	19.7	1.4	9.9	3.2
FMO4	1:171322878	rs2076322 intronic	A>G	24.3	21.2	26.9	24.6	1.4	12.6	0.5
FMO5	1:147209857	rs7541245 intronic	C>A	5.8	6.1	6.0	4.9	0	3.3	0
SLC22A3	6:160451754	rs2076828 3 UTR	C>G	34.2	48.0	51.4	40.2	52.9	37.4	52.2
SLC47A1	17: 19560030	rs2289669 intronic	G>A	2.9	2.0	0	9.8	37.0	45.1	58.1
SLC22A1	6:160154805	rs34059508 Missense	G > A	0	0	0	0	0	3.8	0
ABCC8 KCNJ11	11:17388025	rs5219 Missense	T>C	0	0.5	0	13.9	33.2	26.4	22.6
SLC22A3	6:160437122	rs8187725 Missense	C > T	0	0	0	0	0	0	0

*. - The MAF of all the SNPs in Luhya, Yoruba and African American was taken from Data from the 1000genomes project (http://www.internationalgenome.org), the Pharmacogenomics Knowledgebase (http://www.pharmgkb.org), the UCSF-PMT. (http://www.pharmacogenetica.usfc.edu/) database and NCBI-SNP database (http://www.ncbi.nih.gov). DbSNP - a database of single nucleotide polymorphisms number (http://www.ncbi.nlm.nih.gov/snp), M - Major allele, m - Minor allele, MAF - Minor allele frequency. Bantu (Xhosa, Zulu and Swati).

Table 3.3. Hardy-Weinberg chi-square test for nineteen genetic variants in the Bantu population

Locus	DF	X^2	HWE *p*
rs10213440	1	1.538	0.215
rs11212617	1	0.221	0.638
rs12208357	1	0.002	0.966
rs12752688	1	1.938	0.164
rs12943590	1	1.054	0.305
rs1920145	1	**4.730**	**0.043**
rs2076322	1	0.975	0.323
rs2076828	1	**4.730**	**0.030**
rs2282143	1	0.835	0.361
rs2289669	1	0.124	0.725
rs2617102	1	**9.475**	**0.002**
rs34059508		Monomorphic	
rs34399035		Monomorphic	
rs5219		Monomorphic	
rs594709	1	2.093	0.148
rs7541245	1	0.699	0.403
rs784888	1	1.655	0.198
rs784892	1	0.019	0.890
rs8187725		Monomorphic	

HWE - Hardy-Weinberg equilibrium, statistically significant is shown in bold (X^2 – ChiSq: <3.84, *p*-value - <0.05)

3.4.3. Genetic association analysis of metformin response

The analysis of the association between SNPs and response to metformin therapy for diabetes in Bantu patients is shown in **Table 3.4**. The table presents the associations between alleles, as well as genotypes, and response to metformin measured using odds ratios (ORs), 95% confidence interval (95% CI) and *p*-value derived from unconditional logistic regression. P-value <0.05 was considered significant.

Four out of six missense SNPs (i.e. rs34059508, rs34399035, rs5219 and rs8187725) were monomorphic in this study population (**Table 3.2., Table 3.3., and Table 3.4.**). The *SLC22A1* rs12208357, *SLC22A1* rs2282143 and *SLC47A1* rs2289669 displayed only heterozygous genotypes within the study population (**Table 3.4**). However, for the above-mentioned variant SNPs, none of these heterozygous genotypes were significantly associated with metformin response (p>0.05). The CT genotype of the *FMO2* rs12752688 polymorphism was significantly associated with increased response to metformin therapy (OR= 0.33, 95% CI [0.16-0.68], p-value= 0.003). A moderate association was also found between the GA genotype of *SLC47A2* rs12943590 and a decreased response to metformin therapy (OR= 2.29, 95% CI [1.01-5.21], p-value=0.048 for the heterozygous GA genotype (**Table 3.4**). No significant associations were found between the remaining SNPs and response to metformin treatment for the study population (**Table 3.4**).

Table 3.4. Association between SNPs and responsiveness of metformin therapy for diabetic Bantu patients.

SNP ID			Responders n (%)	Nonresponders n (%)	OR (95% *CI*)	*P*-value
rs10213440	Genotype	CC	3 (5.7)	6 (7.0)	1 (Reference)	
		TC	18 (40.0)	26 (30.2)	0.72 (0.16-3.27)	0.673
		TT	36 (60.3)	54 (62.8)	0.75 (0.18-3.19)	0.697
	Allele	C	21 (19.8)	38 (22.1)	1 (Reference)	
		T	87 (80.2)	134 (77.9)	0.85 (0.47-1.55)	0.597
rs11212617	Genotype	CC	36 (67.9)	60 (71.4)	1 (Reference)	
		AC	16 (30.2)	21 (25.0)	0.79 (0.36-1.70)	0.543
		AA	1 (1.9)	3 (3.6)	1.80 (0.18-17.96)	0.617
	Allele	C	88 (83.0)	141 (83.9)	1 (Reference)	
		A	18 (17.0)	27 (16.1)	0.94 (0.49-1.80)	0.843
rs12208357	Genotype	CC	53 (100)	85 (98.8)	1 (Reference)	
		CT	0	1 (1.2)	1.88 (0.08-46.93)	0.701
	Allele	C	106 (100)	171 (99.4)	1 (Reference)	
		T	0	1 (0.6)	1.86 (0.08-46.15)	0.704
rs12943590	Genotype	GG	42 (79.2)	55 (63.2)	1 (Reference)	
		GA	10 (18.9)	30 (35.6)	2.29 (1.01-5.21)	**0.048**
		AA	1 (1.9)	1 (1.1)	0.76 (0.05 – 12.57)	0.850
	Allele	G	94 (88.7)	140 (81.4)	1 (Reference)	
		A	12 (11.3)	32 (18.6)	1.79 (0.88-3.65)	0.109
rs2617102	Genotype	AA	45 (83.9)	65 (76.5)	1 (Reference)	
		AC	8 (16.1)	14 (16.5)	1.21 (0.47-3.13)	0.692
		CC	0	6 (7.0)	9.03 (0.50-164.33)	0.137
	Allele	A	98 (92.5)	144 (84.7)	1 (Reference)	
		C	8 (7.5)	26 (15.3)	2.21 (0.96-5.09)	0.062
rs594709	Genotype	AA	24 (45.3)	45 (52.3)	1 (Reference)	
		AG	27 (50.9)	36 (41.9)	0.71 (0.35-1.44)	0.342
		GG	2 (3.8)	5 (5.8)	1.33 (0.24-7.39)	0.742

	Allele	A	75 (70.8)	126 (73.3)	1 (Reference)	
		G	31 (29.2)	46 (26.7)	0.88 (0.52-1.51)	0.651
rs12752688	Genotype	CC	24 (45.3)	62 (72.1)	1 (Reference)	
		CT	27 (50.9)	23 (26.7)	0.33 (0.16-0.68)	**0.003**
		TT	2 (3.8)	1 (1.2)	0.19 (0.02-2.23)	0.188
	Allele	C	75 (70.8)	147 (85.5)	1 (Reference)	
		T	31 (29.2)	25 (14.5)	0.41 (0.23-0.75)	**0.004**
rs784888	Genotype	GG	17 (39.5)	26 (34.2)	1 (Reference)	
		CG	18 (41.9)	34 (44.7)	1.24 (0.43-2.85)	0.621
		CC	8 (18.6)	16 (21.1)	1.31 (0.46-3.72)	0.615
	Allele	G	52 (60.5)	86 (57.6)	1 (Reference)	
		C	34 (39.5)	66 (43.4)	1.17 (0.69-2.01)	0.560
rs784892	Genotype	GG	23 (43.4)	34 (39.5)	1 (Reference)	
		AA	7 (13.2)	11 (12.8)	1.06 (0.36-3.15)	0.912
		AG	23 (43.4)	41 (47.7)	1.21 (0.58-2.52)	0.612
	Allele	G	69 (65.1)	109 (63.4)	1 (Reference)	
		A	37 (34.9)	63 (36.6)	1.08 (0.65-1.79)	0.771
rs2289669	Genotype	GG	50 (94.3)	80 (95.2)	1 (Reference)	
		AG	3 (5.7)	4 (4.8)	0.83 (0.18-3.88)	0.816
	Allele	G	103 (97.2)	164 (97.6)	1 (Reference)	
		A	3 (2.8)	4 (2.4)	0.84 (0.18-3.82)	0.819
rs2076828	Genotype	CC	23 (43.4)	45 (52.3)	1 (Reference)	
		CG	21 (39.6)	28 (32.6)	0.68 (0.32-1.45)	0.320
		GG	9 (17.0)	13 (15.1)	0.74 (0.28-1.98)	0.547
	Allele	C	67 (63.2)	118 (68.6)	1 (Reference)	
		G	39 (36.8)	54 (31.4)	0.786 (0.47-1.31)	0.355
rs2076322	Genotype	AA	25 (47.2)	52 (62.7)	1 (Reference)	
		AG	25 (47.2)	30 (36.1)	0.58 (0.28-1.18)	0.131
		GG	3 (5.6)	3 (3.6)	0.48 (0.09-2.55)	0.390
	Allele	A	75 (70.8)	134 (78.8)	1 (Reference)	
		G	31 (29.2)	36 (21.2)	0.65 (0.37-1.13)	0.130

rs2282143	Genotype	CC	44 (83.0)	75 (87.2)	1 (Reference)	
		CT	9 (17.0)	11 (12.8)	0.72 (0.28-1.87)	0.495
	Allele	C	97 (91.5)	161 (93.3)	1 (Reference)	
		T	9 (8.5)	11 (6.7)	0.74 (0.29-1.84)	0.513
rs1920145	Genotype	CC	10 (18.9)	25 (29.8)	1 (Reference)	
		CT	34 (64.2)	47 (51.1)	0.55 (0.23-1.30)	0.175
		TT	9 (16.9)	15 (19.1)	0.67 (0.22-2.01)	0.472
	Allele	C	54 (50.9)	96 (55.7)	1 (Reference)	
		T	52 (49.1)	76 (44.3)	0.82 (0.51-1.34)	0.429
rs34059508	Genotype	GG	53 (100)	87 (100)	-	
	Allele	G	106 (100)	174 (100)	-	
rs34399035	Genotype	CC	53 (100)	88 (100)	-	
	Allele	C	106 (100)	176 (100)	-	
rs5219	Genotype	CC	51 (100)	83 (100)	-	
	Allele	C	102 (100)	166 (100)	-	
rs7541245	Genotype	CC	48 (90.6)	75 (88.2)	1 (Reference)	
		AC	5 (9.4)	9 (10.6)	1.15 (0.36-3.64)	0.810
		AA	0	1 (1.2)	1.93 (0.08-48.28)	0.690
	Allele	C	101 (95.3)	159 (93.6)	1 (Reference)	
		A	5 (4.7)	11 (6.4)	1.40 (0.47-4.14)	0.546
rs8187725	Genotype	CC	53 (100)	86 (100)	-	
	Allele	C	106 (100)	172 (100)	-	

OR – odds ratio, *CI* – confidence interval, 1 (Reference) – wild type (ancestral), statistically significant (p-value<0.05).

3.5. Discussion

The aim of the study was to investigate the genetic association between nineteen pharmacogenomics biomarkers (SNPs) and response to metformin treatment, and to evaluate their suitability for individualizing metformin therapy for diabetic patients. Two multiplex MassARRAY systems (Agena Bioscience™) were designed and optimized by Inqaba Biotechnical Industries (Pretoria, South Africa), and used for the genotyping of the selected SNPs for 140 T2DM outpatients from the Bantu population group. The cohort of patients was divided into two groups; patients with controlled (responsive) and patients with uncontrolled (nonresponsive) diabetes following the treatment with metformin. The HbA1c was used as a marker for treatment response (**Figure 3.1**). In previous studies, the targeted treatment for a response for diabetes has been defined in a number of ways. According to Shikata *et al*. (2007), Umamaheswaran *et al*. (2014) and Sherifali *et al*. (2010) their treatment success was defined as ability to reach treatment target by 0.5 – 1.5% HbA1c reduction (Shikata *et al*, 2007; Umamaheswaran *et al*, 2014; Sherifali *et al*, 2010). In some studies, the treatment success was defined as the ability to achieve HbA1c≤7% treatment target (Becker *et al*, 2009a; Florez *et al*, 2012; Godarts *et al*, 2015; Van Leeuwen *et al*, 2012). For this study, considering that some patients had hypertension, the treatment target was defined as follows: HbA1c ≤8% for responders and HbA1c ≥8% for non-responders as suggested by the study done by Kashki *et al*. (2015). This is also accepted by clinical guidelines due to other comorbidities e.g. hypertension and dyslipidemia (Kashki *et al*, 2015; CDE, 2018; Amod, 2017).

Metformin is often the first drug used to treat newly diagnosed type 2 diabetic patients, and it is widely prescribed worldwide. Metformin is effective as monotherapy and in combination with nearly every other therapy for type 2 diabetes, and its utility is supported by data from a large number of clinical trials (Sanchez and Inzucchi, 2017; Maruthur *et al*, 2016). However, despite its exceptional efficacy and safety profile, about 38% of type 2 diabetes patients who have taken metformin failed to reach target fasting glucose level (Reitman and Schadt, 2007). Recent studies suggest that interpatient variability in response to metformin therapy could be related to polymorphisms in the OCT genes and/or the MATE genes, as well as other genetic variants (Avery *et al*, 2009).

In the present study, nineteen SNPs with some clinical evidence of association with response to metformin treatment for T2DM were selected using the Pharmacogenomics knowledge base (www.pharmgkb.org), Ensembl (http://www.Ensembl.org), as well as an extensive survey of literature. In addition to the association analysis, the observed MAF for the SNPs were also compared to world populations i.e. Luhya, Yoruba, African American, Japanese, British and Chinese Dai. All the SNPs showed no deviation from HWE except for rs2617102 (**Table 3.2**). Among all the genetic variants included in the study, there was a significant association between responsiveness to metformin therapy for only for 2 SNPs; *FMO2* rs12752688 and *SLC47A2* rs12943590 (**Table 3.4**).

An investigation conducted by Breitenstein *et al*, (2015) on variants cross FMO genes (rs13376631, rs12752688, rs1920145 and rs7541245), has shown no association was between FMOs variants and glycaemic response of metformin except for *FMO5* rs7541245 (Breitenstein *et al*, 2015). Nevertheless, this study has identified a potential role of *FMO2* rs12752688 C/T in metformin efficacy (**Table 3.4**). This variant rs12752688 is a known rare SNP in the FMO gene and is majorly expressed in lungs and fat cells in most mammals and other non-human primates, but rarely in the human populations (Hines *et al*, 2003; Yueh *et al*, 1997; Dolphin *et al*, 1998; Krueger *et al*, 2001). However, because of its intergenic location its exact role is not clear. This suggests that more studies are needed to validate these findings, imperative to elucidate the relationship between the FMO genes' function and T2DM, as well as to define these enzymes' clinical relevance.

The promoter variant *SLC47A2* rs12943590 showed a considerable influence on metformin response (**Table 3.4**). A recent study conducted by Phani *et al*, (2018) has shown that this variant is expected to alter function or expression of the *SLC47A2* transporter gene (Phani *et al*, 2018). In addition, according to a study conducted by Choi, 2011, on unrelated healthy patients from four major ethnic groups (i.e. European Americans, Africans American, Chinese Americans and Mexican Americans), *SLC47A2* rs12943590 showed a significant association with poor glycaemic response to metformin (Choi *et al*, 2011). Others studies have demonstrated that this variant is associated with poor plasma glucose control of metformin when assessed by differences in HbA1c levels (Stocker *et al*, 2013).

The *KCNJ11* variant rs5219 has been showed to have clinical relevance on both metformin and SUs drug response in diabetic patients. Conflicting results were reported by other studies (Sesti *et al*, 2006, Gloyn *et al*, 2001, Holstein and Beil, 2009, Pearson *et al*, 2006; Siklar *et al*, 2011). Sesti *et al* (2006) showed that patients with the rs5219 variant had a higher probability of treatment failure when treated with a combination of SU and metformin (Sesti *et al*, 2006). Another study done on this variant (rs5219) showed a reduced response to SU in carriers of *KCNJ11* 23Lys coding allele thus increasing HbA1c level (Holstein and Beil, 2009). A prior study done by Gloyn *et al.* (2001) on the Caucasian population, however, showed no association to the SU therapy (Gloyn *et al*, 2001; Holstein and Beil, 2009). Moreover, other studies have reported that diabetic patients with this variant respond better to pharmacotherapy with SUs as compared to insulin (Pearson *et al*, 2006; Siklar *et al*, 2011). For this study rs5219 was observed to be monomorphic (**Table 3.2 and 3.3**). Therefore, no conclusion could be made regarding the association between this SNP and response to metformin treatment.

The role of OCT3 rs8187725 was investigated in the present study and was found to be monomorphic in the study cohort, as well as in the other Sub-Saharan African populations (**Table 3.3**). This is a rare variant and in some studies, it was shown to have a very low allele frequency (Sakata *et al*, 2010; Chen *et al*, 2010c). A study conducted by Jacobs, (2014) showed similar findings on Xhosa and Cape Coloured populations; where this variant was also not observed (Jacobs, 2014 Ph.D. thesis). However, in other studies, it has been associated with the pharmacokinetics of

metformin in healthy individuals (Tzvetkov *et al*, 2001). Chen *et al*. (2010b) have also successfully replicated this association (Chen *et al*, 2010b). Given the prevalence of T2DM in South Africa and the widespread use of metformin as a therapeutic, the distribution of this variant in the indigenous African populations requires further investigation. The *in vivo* effect of OCT3 rs8187725 on metformin pharmacokinetics and efficacy must be assessed when identified in other South African and/or any other indigenous African population since it has not yet been demonstrated.

3.6. Summary

A total of 117 Xhosa T2DM outpatients were recruited from Cecilia Makiwane Hospital (**Chapter 2**) and only 81 T2DM patients were on metformin monotherapy. Due to small sample size, 49 Zulu and 10 Swati T2DM patients were included to include a total of 140 T2DM patients in the present study. The focus of this study was to investigate the genetic association between nineteen pharmacogenomics biomarkers (SNPs) and response to metformin treatment and to evaluate their suitability for individualizing metformin therapy for T2DM patients from indigenous Bantu populations of South Africa. The *FMO2* rs12752688 polymorphism was significantly associated with increased response to metformin therapy. A moderate association was also found between *SLC47A2* rs12943590 and a decreased response to treatment with metformin for T2DM. Therefore, it is suggested that the *FMO2* rs12752688 polymorphism should be included in pharmacogenomics profiling systems developed to individualize metformin therapy for diabetic patients from the Bantu populations.

Chapter Four

Conclusion and future prospectus

South Africa is one of the countries experiencing an increasing burden of NCDs. NCDs are the major source of mortality and morbidity, which is estimated to surpass the burden of infectious diseases by 2035. The two most common NCDs associated with rapid mortality increase are DM and HTN. They frequently occur in the same individuals in clinical practice. Both of these diseases, i.e. DM and HTN, can be a result of a combination of modifiable risk factors (behavioral factors) and non-modifiable risk factors (genetic, physiological, and environmental). The burden of NCDs in South Africa is predicted to increase substantially in the next decades if the necessary preventative measures are not taken. Therefore new strategies are needed to effectively manage these diseases, which include addressing both modifiable and non-modifiable risk factors for patients with NCDs.

Diabetes mellitus and hypertension have been identified as one of the leading causes for the rise in non-communicable diseases worldwide. The four major risk factors contributing to the NCDs burden are: tobacco use, physical inactivity, unhealthy diets and alcohol consumption. Insight into the effects that risk factors have on NCDs such as diabetes mellitus and hypertension is crucial for effective management and treatment of these diseases in under-studied populations. In addition, numerous studies have reported the involvement of genetic polymorphisms and their interactions in genetic pathways (i.e. pharmacodynamics and pharmacokinetics), in treatment outcomes, predominantly observed metformin therapy. Despite its widespread use, there is considerable variation in response to

metformin; with more than one-third of the patients failing to achieve adequate glycaemic control. The aim of the current study was to develop and validate a pharmacogenomics profiling panel suitable for the individualizing metformin therapy for patients from the Bantu populations in South Africa.

As a preliminary measure, the prevalence of risk factors for DM and HTN in South Africa was explored within an economically disadvantaged population group. The extent of uncontrolled DM and HTN was investigated among 140 resource- constrained patients receiving treatment in rural areas. A significant burden of DM and HTN was observed where a reduced risk of DM was associated with no "salt intake", "never smoke", and normal levels of TG and HDL whilst a reduced risk of hypertension was associated with decreased BMI. In order to reduce the burden of NCDs, the development of best practices for affordable and effective programs in screening, prevention, detection and treatment of DM and HTN is essential. In addition, comprehensive intervention strategies should be implemented across the country.

Subsequently, nineteen pharmacogenomics biomarkers were evaluated for their suitability for individualized metformin therapy for T2DM Patients. A genetic association study was conducted to investigate the level of association between nineteen pharmacogenomics biomarkers (SNPs) and response to metformin treatment, and to evaluate their suitability for individualizing metformin therapy for diabetic patients from the Bantu populations. The individualization of metformin therapy has the potential to reduce the incidence of uncontrolled T2DM among patients taking this first-line anti-diabetic drug.

The *FMO2* rs12752688 polymorphism was significantly associated with increased response to metformin therapy. A moderate association was also found between *SLC47A2* rs12943590 and a decreased response to treatment with metformin for T2DM. To our knowledge, this is the first study that investigated the association between genetic variants and responsiveness to medication for diabetic patients from the indigenous Bantu population of South Africa. The *FMO2* rs12752688 polymorphism is suggested to be included in pharmacogenomics profiling systems developed to individualize metformin therapy for diabetic patients from the Bantu populations. It may be concluded that better assessment of disease risk, further understanding of disease mechanisms, and the optimization of therapy are paramount to reduce the burden of DM and HTN in South Africa, and worldwide.

The following limitations need to be considered; the cross-sectional design and recruitment of participants from one study center might have limited the generalization of the findings. An inconclusive report was observed due to the lack of adequate glycemic control amongst a large number of patients within the 12 months of study and the different use of HbA1c baselines definition obtained from many similar studies. Moreover, the genetic risk guide in the future must be improved; the definition of DM and classification of subtypes of DM should be more precise. Poor adherence displayed by the T2DM patients prescribed with metformin is assumed as a critical challenge resulting in relapse on treatment (unsuccessful treatment). This challenge is a known problem for healthcare professionals. For this study, patient responses were relied upon; however, patients tend to be reluctant to adhere to treatment and are dishonest during healthcare

professionals' follow-ups, which may have affected the results of the study. In addition, the importance of variations in different populations and with the consideration of the diversity in African populations should be highlighted. It is ideal to obtain a large sample scale in identifying the association between polymorphisms and T2DM in a population.

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