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Genetic polymorphisms of Angiotensin-converting enzyme (ACE), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Vascular endothelial growth factor (VEGF) and Angiotensinogen (AGT) associated with contrast induced nephropathy in black South Africans

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Genetic polymorphisms of Angiotensin-converting enzyme (*ACE*), Interleukin-6 (*IL-6*), Interleukin-8 (*IL-8*), Vascular endothelial growth factor (*VEGF*) and Angiotensinogen (*AGT*) associated with contrast induced nephropathy in black South Africans



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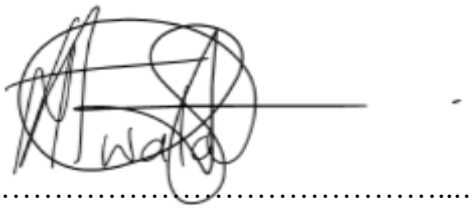
A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

January, 2022

DECLARATION

I, **Nomthandazo Athelia Twala**, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.



……...….

…….30th…...day of……January…….…, 2022.

DEDICATION

To Christina. M. Twala,
my loving mother, Mnyamande,
my pillar of strength

And

In loving memory of my grandfather
Themba. J. Legodi
1938-2018

ABSTRACT

Background: Iatrogenic contrast-induced nephropathy (CIN) is the third leading cause of hospital-acquired acute kidney injury (AKI) in the world, and it remains a major cause of morbidity, increases overall mortality and is associated with prolonged hospital stay and higher medical costs. Despite the positive association of genetic polymorphisms in the *ACE, IL-6, IL-8, VEGF* and *AGT* genes with CIN seen in previous studies, the CIN genetic susceptibility in Sub-Saharan Africa is unknown. Thus, this study was conducted for the purpose of demonstrating genetic association of candidate gene (*IL-6, IL-8, ACE, VEGF*, and *AGT*) polymorphisms with increased CIN in the black population receiving contrast media.

Methods: This retrospective study is based on black South African patients that had undergone enhanced computerized tomography (CT) and angiography procedures. CIN was defined as an increase in serum creatinine >25% or an absolute increase >44 µmol/l from baseline at 48-72 hours after exposure to contrast media. Genomic DNA was extracted from peripheral blood samples using the automated Maxwell® 16 DNA Purification Kit from Promega®. For the samples in which insufficient DNA was extracted, a standard salting out method was used. Single nucleotide polymorphisms (SNPs) in the *IL-6* (rs1800796), *IL-8* (rs4073), *VEGF* (rs3025039 and rs2010963) and *AGT* (rs699) genes were genotyped using a TaqMan® SNP genotyping assay to generate a fluorescent signal during Real-Time PCR. The *ACE* SNP (rs4646994) was genotyped using conventional PCR and the resulting PCR products resolved using agarose gel electrophoresis. Sequencing was performed to confirm the genotype results of *IL-6*, *IL-8*, *VEGF*, and *AGT* SNPs. Outcome variables were the occurrence of CIN and mortality rate.

Results: 228 patients were included in the study, 30 (13%) of whom developed CIN. Statistical analysis using different genetic models (dominant, recessive, multiplicative and additive) indicated no association between *IL-6, IL-8, ACE, VEGF*, and *AGT* gene polymorphisms and the risk of CIN. The *p*-values of all the SNPs were above 0.05. The mortality rate was significantly increased in the CIN+ group compared to the CIN- group (24.1% versus 7.5%; p=0.007).

Conclusion: This study proposes that there is no association between the polymorphisms assessed in the candidate genes and the risk of CIN in the black South African population. The most limiting factor of this study was sample size, since only 13% of the cohort developed CIN. Therefore, further research using a larger sample size is suggested which could provide useful information for the early detection of CIN and guiding directed therapy.

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LIST OF SYMBOLS

bp	base pair
^{o}C	degrees Celsius
g	grams
g/dL	grams per decilitre
$MgCl_2$	Magnesium Chloride
µl	microlitre
µM	micromolar
µmol/L	micromoles per litre
ml	millilitre
mL/min	millilitre per minute
mmHg	millimetre of mercury
mM	millimolar
mmol/L	millimoles per litre
mOsm/Kg H_2O	milliosmoles per kilogram of water
ng	nanogram
ng/µl	nanogram per microlitre
nm	nanometre
nM	nanomolar
rpm	revolutions per minute
Tm	temperature
U	units
U/µl	units per microlitre

LIST OF ABBREVIATIONS

ACE	Angiotensinogen-converting enzyme
ACEI	angiotensin converting enzyme inhibitor
AD	Allelic Discrimination
AGT	Angiotensinogen
AKI	Acute kidney injury
AMI	Acute myocardial infarction
Ang	Angiotensin
Ang II	Angiotensin II
ARB	angiotensin receptor blocker
ARF	Acute renal failure
ARI	Acute renal insufficiency
ATP	adenosine triphosphate
CAR	Canadian Association of Radiologists
CHF	Congestive heart failure
CI	Confidence interval
CIN	Contrast-Induced Nephropathy
CKD	Chronic kidney disease
CM	Contrast media
CPB	Cardiopulmonary bypass
CT	Computerized tomography
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ESRD	End stage renal disease
ESUR	European Society of Urogenital Radiology
ET-A	Endothelin-A
GFR	Glomerular filtration rate
HIV	Human immunodeficiency virus

HREC	Human Research Ethics Committee
IABP	Intra-aortic balloon pump
IDT	Integrated DNA Technologies
IL-6	Interleukin- 6
IL-8	Interleukin- 8
IL-10	Interleukin- 10
IQR	Interquartile range
KDIGO	Kidney Disease Improving Global Outcomes
LVEF	Left ventricular ejection fraction
MAF	Minor allele frequency
NAC	N-acetyl cysteine
NCBI	National Center for Biotechnology Information
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
NTC	Non-template control
PGE2	Prostaglandin
PGI2	Prostacyclin
PCI	Percutaneous coronary interventions
PCR	Polymerase chain reaction
RBF	Renal blood flow
ROS	Reactive oxygen species
RCT	Randomized clinical trial
RAS	renin-angiotensin system
RRT	Renal replacement therapy
RFU	Relative fluorescent units
RefSeq	Reference sequences
SNP	Single nucleotide polymorphisms
TNFα	Tumour necrosis factor α
uAnCR	urine angiotensinogen/creatinine ratio
UTR	untranslated region
VEGF	Vascular endothelial growth factor

Chapter 1

1. INTRODUCTION

Iatrogenic contrast-induced nephropathy (CIN) is the third leading cause of hospital-acquired acute kidney injury (AKI) in the world, accounting for 10% of all hospital-acquired kidney injury cases (Shema *et al.,* 2009; Evola *et al.,* 2012). Despite current knowledge about risk factors of CIN and preventive guidelines, it remains a major cause of morbidity, increases overall mortality and is associated with prolonged hospital stays and higher medical costs (Shema *et al*., 2009; Mitchell *et al*., 2010; Evola *et al*., 2012; Chao, 2013; Chalikias *et al.,* 2016). Globally, it is estimated that the number of iodinated contrast media doses given per year is approximately 75 million, administered for both diagnostic and therapeutic purposes (Pasternak & Williamson, 2012; Mitchell *et al.*, 2013). According to Solomon (2014), rates of computed tomography (CT) procedures using iodinated contrast media are almost 30 million per year and rates of angiography exceed 2 million per year in developed countries. Among the radiological procedures using contrast media, percutaneous coronary interventions (PCI) and coronary angiography are associated with the highest rates of CIN (Nash *et al*., 2002; Mehran & Nikolsky, 2006).

A study by Banda *et al.* (2016) showed that, like in developed countries, the use of contrast media during radiological procedures resulted in high incidence of CIN, 16.4% in sub-Saharan Africa. In our setting, CIN is a potential complication of increased iodinated contrast media administration (Perrin *et al.,* 2012).

1.1. Contrast induced nephropathy

Contrast induced nephropathy, an iatrogenic disorder, is defined as an acute decline in renal function following the administration of iodinated contrast media (Thomsen & Morcos, 2003; Nickolas *et al*., 2008; Shema *et al*., 2009; Heyman *et al*., 2010; Chang *et al*., 2013; Meinel *et al*., 2014). Contrast induced nephropathy is diagnosed based on three factors: an absolute or relative rise in serum creatinine from baseline, exposure to contrast media and exclusion of other confounders for renal disease (Mehran & Nikolsky, 2006).

How the change in serum creatinine is defined in CIN can vary. The European Society of Urogenital Radiology (ESUR) defines CIN as a rise in serum creatinine levels of more than 25% or 44 µmol/l from baseline within 48-72 hours following a radiological procedure that exposes the

body to iodinated contrast media (Thomsen & Morcos, 2003; Mehran & Nikolsky, 2006; Shema *et al*., 2009; Narula *et al*., 2014; Chalikias *et al*., 2016). This definition of CIN has predicted early and late mortality as well as harmful cardiovascular events (Bellin *et al*., 2002; Thomsen & Morcos, 2003; Mehran & Nikolsky, 2006). The updated Canadian Association of Radiologists (CAR) guidelines also defines CIN as an increase in serum creatinine of >25% of the baseline value over a 72 hour period (Owen *et al*., 2014). This means that CIN occurs within 1-3 days, peaking 3-5 days after administration of contrast media (Owen *et al*., 2014; Chalikias *et al*., 2016). In a comparison of the absolute (>44 µmol/l) versus relative increase (>25%) in serum creatinine, the Contrast Induced Nephropathy Consensus Working Panel recommended the use of the latter definition (Solomon & Deray, 2006). However, the Kidney Disease Improving Global Outcomes (KDIGO) defines CIN as a rise in serum creatinine levels by >26.5 µmol/l occuring within 48 hours of contrast administration or a rise in serum creatinine by 50% within 7 days (Khwaja, 2012).

1.2. Pathophysiology of CIN

Although the pathophysiology of CIN is complex and not well-understood, multiple mechanisms by which contrast agents cause AKI have been proposed. The two main distinct but interacting mechanisms include disturbances in renal blood flow (hemodynamic effects), and direct renal tubular cell cytotoxicity (Heyman *et al*., 2008; Berg, 2000; Rudnick & Goldfarb, 2003; Parfrey, 2005; Persson *et al*., 2005; Pucelikova *et al*., 2008; Geenen *et al*., 2013; Mohammed *et al*., 2013; Hossain *et al*., 2018; Mehran *et al*., 2019). The mechanisms of CIN are depicted in Figure 1.1.

1.2.1. Hemodynamic effects

The direct intra-arterial injection of contrast media results in a biphasic effect on renal blood flow (RBF), with an initial brief increase in blood flow (vasodilation) by 20% to the renal cortex followed by a 40% decrease in blood flow to the outer renal medulla resulting in vasoconstriction (Nygren, 1992; Berg, 2000; Katzberg, 2005). According to Rudnick and Goldfarb, the renal medulla is susceptible to hypoxic injury from factors that reduce RBF due to its high metabolic activity, such as the process of sodium reabsorption which requires higher levels of oxygen (Brezis & Rosen, 1995; Rudnick & Goldfarb, 2003; Geenen *et al*., 2013).The oxygen supply to the renal medulla is further decreased due to an increase in oxygen demand required for contrast media excretion which causes medulla ischemia (Parfrey, 2005).

The reduction in RBF results from the response of vasoactive substances (endogenous biochemical factors) to the administration of contrast medium (Berg, 2000; Katzberg, 2005). These endogenous factors include endothelin, reactive oxygen species (ROS), adenosine, nitric oxide (NO), prostaglandins, and calcium ions (Berg, 2000; Heyman *et al*., 2005; Geenen *et al*., 2013). Renal vasoconstriction is induced by an increase in endothelin through the activation of the endothelin-A (ET-A) receptor (Wong *et al*., 2012; Yang *et al*., 2018). In addition, according to Yang *et al*. (2018), contrast medium stimulates vasoconstriction by deflecting blood flow from the medulla to the cortex resulting in the reduction of Glomerular filtration rate (GFR), followed by renal medulla hypoxia (Persson *et al*., 2005).

The decline in RBF leads to an increase in the generation and release of ROS by oxidative stress (Geenen *et al*., 2013; Hossain *et al*., 2018; Yang *et al*., 2018). Reactive oxygen species trigger and induce vasoconstriction by increasing angiotensinogen-II and endothelin-I vasoconstrictors, and decreasing vasodilative NO (Heyman *et al*., 2010). According to Hossain and colleagues, a group of investigators using animal models illustrated the significance of calcium influx in the process of renal vasoconstriction after the administration of contrast (Hossain *et al*., 2018).

Renal vasodilation is stimulated by adenosine through the formation of NO, mediated by the adenosine A2 receptor (Heyman *et al.,* 2010). However, a seminal study conducted by Klause and colleagues demonstrated increased levels of adenosine induced vasoconstriction (Klause *et al*., 1998; Yang *et al*., 2018). Furthermore, Myers *et al*. (2006), found that the reduction of blood flow to the cortex and medulla were caused by a decrease in prostaglandin (PGE2) and endogenous medullary and cortical NO synthesis in rat kidneys, resulting in the suppression of renal vasodilation. Prostaglandin produces prostacyclin (PGI2), an effective vasodilator which induces renal vasodilation, which is suppressed in CIN positive individuals (Wong *et al*., 2012). In addition, endogenous endothelin, angiotensin II, and hypoxia trigger the release of NO from endothelium which, along with prostaglandin, decreases sodium reabsorption in all tubular segments for NO and in the collecting duct for prostaglandin, resulting in medullary ischemia (Wong *et al*., 2012).

1.2.2 Direct renal tubular cell cytotoxicity

Iodinated contrast media directly induces renal tubular (epithelial) cell damage, associated with higher levels of intracellular ROS, which increases vasoconstrictive mediators resulting in renal vasoconstriction and tubular cell apoptosis (Haller & Hizoh, 2004; Persson *et al*., 2005; Mamoulakis *et al*., 2017). Reactive oxygen species produced by contrast media induces renal injury which leads to compromised RBF and a decrease in GFR (Persson *et al*., 2005). Additional cytotoxic effects of contrast media, as described by Haller and Hizoh, include an increase in calcium content inducing vasoconstriction, cellular energy metabolism change, intercellular junction disruptions and alterations in epithelial cell polarity (Haller & Hizoh, 2004; Hossain *et al*., 2018). The contrast agents cause a reduction in potassium and adenosine triphosphate (ATP) resulting in cellular energy failure (Haller & Hizoh, 2004). Furthermore, the radiocontrast agents open or weaken the intercellular junctions causing disturbances of the polarized epithelial cell surface (Haller *et al*., 1997).

Greenen and colleagues concluded that tubular cell necrosis was a consequence of interacting pathways, including ROS generation, which enhance the effect of vasoconstrictors and reduce vasodilators, ROS formation resulting in epithelial cell injury, and the direct toxic effect of contrast media on epithelial cells leading to an increase in the generation of ROS (Geenen *et al*., 2013).

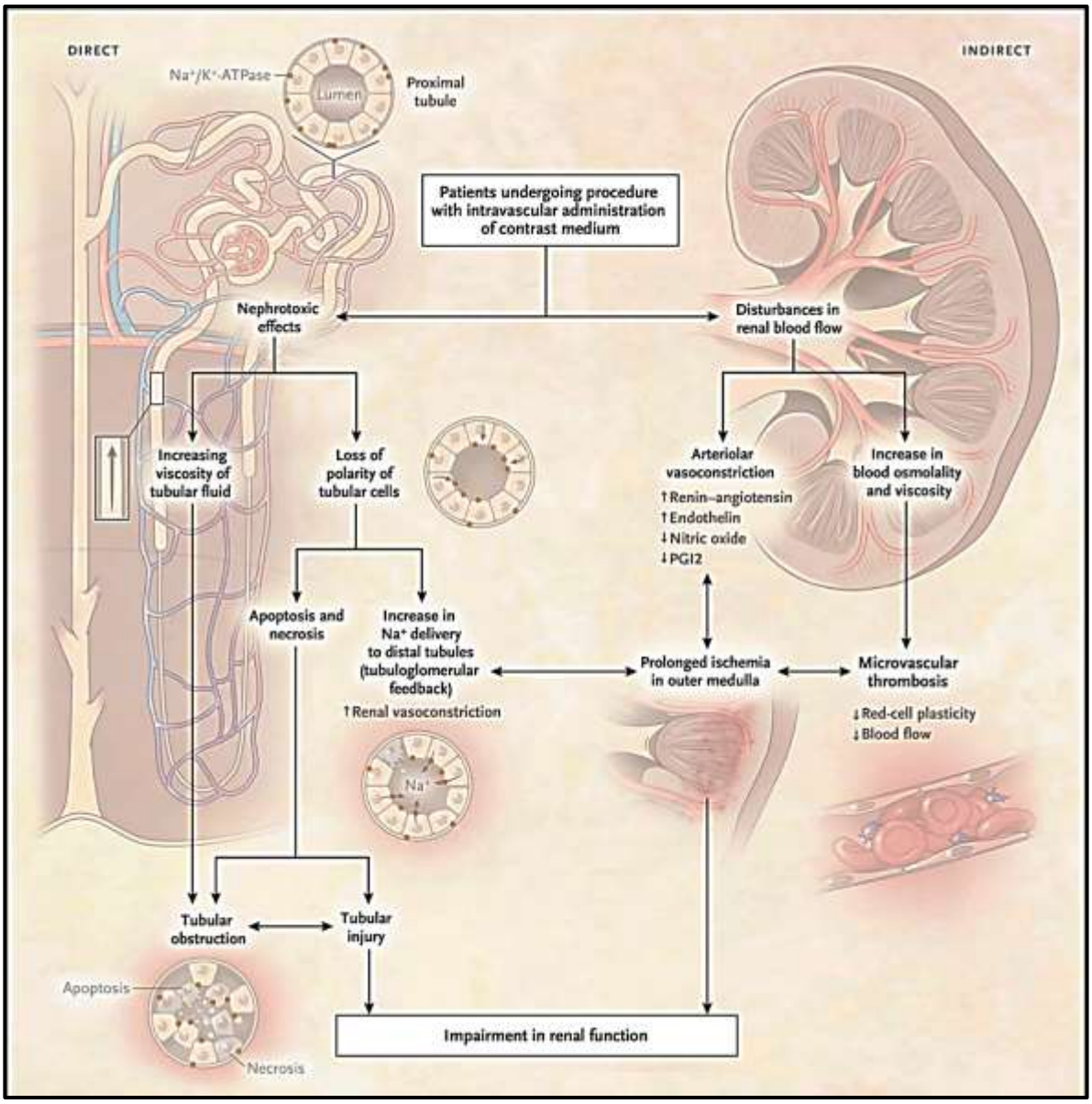

Figure 1.1: Proposed Mechanisms of Contrast-Associated AKI (CIN Pathophysiology).

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1.3. Epidemiology of CIN

The incidence of CIN varies widely, depending on the definition of CIN used, CIN risk factors, and the volume and type of contrast media used (Berg, 2000; Marenzi *et al.,* 2003; Lameire, 2006; Thomsen *et al.,* 2008). Furthermore, the overall incidence of CIN is reported to be 1- 6% in the general population and up to 50% (or higher) among risk groups with advanced kidney disease (Parfrey, 2005; Mehran & Nikolsky, 2006; Thomsen *et al*., 2008).

In a prospective cohort study conducted by Banda and colleagues at the Charlotte Maxeke Hospital in Johannesburg, the incidence of CIN was 16.4% (61/371) among the study patients undergoing CT scan contrast media administration when based on a relative rise of >25% in serum creatinine and 4.6% based on an increase in serum creatinine of >44 µmol/l post contrast media administration (Banda *et al*., 2016). Similarly, Shema and co-workers reported a 4.5% prevalence of CIN among hospitalized Israeli patients when based on an increase in serum creatinine of >44 µmol/l within five days post contrast media administration. However, higher rates of 14.1- 21.1% of CIN were observed among selected risk groups such as patients with renal insufficiency (Shema *et al.,* 2009).

According to Shema and colleagues, CIN has a higher prevalence in patients with comorbidities such as sepsis, chronic kidney disease, heart failure, cancer, ischemic heart disease, diabetes, and hypertension (Shema *et al.,* 2009). Patients with diabetic nephropathy undergoing coronary angiography had a higher incidence of CIN, about 50% (Thomsen & Morcos, 2003). Shema *et al*. (2009), demonstrated a high rate of CIN, about 44% prevalence in patients who had both diabetes and renal insufficiency. This is supported by an earlier study comparing CIN susceptibility among patients with diabetes mellitus or pre-existing renal insufficiency, to patients with both renal impairment and diabetes following contrast administration (Parfrey *et al*., 1989; Parfrey, 2005). CIN development occurred more frequently amongst patients with both diabetes and renal impairment, 4-fold higher than in diabetic patients or patients with pre-existing renal insufficiency alone (Parfrey *et al*., 1989; Katzberg & Newhouse, 2010). In a prospective case-controlled study of cardiac patients undergoing percutaneous coronary intervention (PCI) in Taiwan, prevalence of CIN was 10.3% (Chang *et al.,* 2013). In this study, CIN was defined as an increased serum creatinine of ≥ 44 µmol/l from baseline (Chang *et al.,* 2013). In other studies, Mitchell *et al*. (2010) reported a 14% prevalence of CIN among patients with suspected pulmonary embolism in

Northern Carolina, USA and Narula and colleagues conducted a randomized clinical trial (RCT) study in the USA which reported a 16.1% rate of CIN among PCI patients (Narula *et al*., 2014).

1.4. Risk Factors associated with CIN

The factors increasing the risk of CIN development include advanced age, pre-existing impairment of renal function, comorbidities and volume and type (osmolarity) of contrast media (Morcos *et al.,* 1999; Bellin *et al*., 2002; Mehran & Nikolsky, 2006). These risk factors have been investigated in several studies and can be summarized into two categories: patient level (non-modifiable) and procedure level (modifiable) factors (Table 1.1).

Table 1.1 Risk factors for the development of CIN

Fixed (non-modifiable) risk factors	**Modifiable risk factors**
Older age	Volume of CM
Diabetes mellitus	Hypotension
Pre-existing renal failure	Anaemia and blood loss
Advanced CHF	Dehydration
Low LVEF	Low serum albumin level (<35 g/l)
Acute myocardial infarction	ACE inhibitors
Cardiogenic shock	Diuretics
Renal transplant	Non-steroidal anti-inflammatory
	Nephrotoxic antibiotics
	IABP

ACE, angiotensin-converting enzyme; CM, contrast media; IABP, intra-aortic balloon pump; CHF, congestive heart failure; CIN, contrast-induced nephropathy; LVEF, left ventricular ejection fraction. Reproduced with permission from Mehran and Nikolsky (2006).

1.4.1. Patient level CIN risk factors

1.4.1.1. Older age

In many previous studies, advanced age was reported as an important predictor of CIN development, particularly in patients older than 75 years (Parfrey *et al*., 1989; Mehran & Nikolsky, 2006). The definition of advanced age varies among studies; however, a range of over 65-75 years is generally agreed upon (Mehran e*t al*., 2004). The high prevalence of CIN observed in elderly patients are because of age-related changes in renal function, such as the reduction of GFR, tubular secretion, and concentrating ability caused by the dominance of renal vasoconstrictive forces (Mehran & Nikolsky, 2006; Toprak & Cirit, 2006; Pucelikova *et al.*, 2008). Additional factors include vascular stiffness caused by the tortuosity and calcification of vessels, the presence of renovascular diseases and the presence of other comorbidities which likely increase in older patients (Mehran & Nikolsky, 2006; Toprak & Cirit, 2006).

In a prospective study conducted by Rich and Crecelius in which patients older than 70 years were subjected to cardiac catheterization, the incidence of CIN was reported as 11% (Rich & Crecelius, 1990). Other studies have reported patients older than 75 years as having a 2-5-fold increased risk of developing CIN with an estimation of 2% increased risk with every one-year increment after 75 years (Rihal *et al*., 2002; Marenzi e*t al*., 2004; Mehran e*t al*., 2004). Furthermore, Kohli and colleagues illustrated the significance of age in CIN development by screening patients older than 60 years over a 12-month period for the development of acute renal insufficiency (ARI). The results demonstrated a 17% risk of CIN in elderly (>60 years) patients compared to a 4% risk in younger (<60 years) patients (Kohli *et al*., 2000).

1.4.1.2. Pre-existing renal insufficiency

Pre-existing renal insufficiency with high levels of serum creatinine is considered the most important risk factor in CIN development with high rates of CIN observed in patients with pre-existing renal disease (McCullough *et al*., 1997; Rihal *et al*., 2002; Mehran & Nikolsky, 2006). Several studies have illustrated a GFR less than 60 ml/min/1.73 m^2 as a major risk factor for the development of CIN (Mehran *et al*., 2004; McCullough & Soman, 2005). According to Hall *et al*. (1992), CIN incidence was only 2% when baseline plasma creatinine of patients was ≤1.2 mg/dL. Chronic kidney disease (CKD) is associated with a reduced vasodilator response important for the

development of CIN, and renal insufficiency is associated with a slower contrast media clearance than for patients with normal renal function (Toprak & Cirit, 2006).

In a prospective study of 1144 patients who underwent cardiac catheterization, CIN developed in 20% of those with serum creatinine concentration higher than 2.0 mg/dL; a low risk (4.9%) of CIN was documented in patients with normal renal function where creatinine levels did not exceed 1.2 mg/dL (Davidson *et al*., 1989). Similar findings have been reported by other investigators, who found that CIN incidence increased from 5.3% (3232 patients) to 15.7% (959 patients) with baseline serum creatinine concentration greater than 1.2 mg/dL (Nash *et al.,* 2002). In another study, Rihal and colleagues found that CIN developed in 22% of patients who had serum creatinine levels of 2.0 to 2.9 mg/dL and 30% of patients with serum creatinine levels greater than 3.0 mg/dL (Rihal *et al*., 2002).

1.4.1.3. CIN associated comorbidities

Patients with additional comorbidities such as the underlying chronic kidney disease (CKD), diabetes mellitus, and cardiovascular diseases are linked with high risk of developing CIN (Nash *et al*., 2002; Golshahi *et al.,* 2014). Toprak and Cirit associated both CKD and diabetes nephropathy with renal endothelial dysfunction and reduced renal vasodilation (Toprak & Cirit, 2006).

The risk of developing CIN is 4-fold higher in patients with both diabetes and CKD compared to patients without diabetes mellitus (Manske *et al*., 1990). The incidence of CIN in patients with pre-existing renal disease (CKD) varies from 14.8-55% (Mehran & Nikolsky, 2006; Rihal *et al*., 2002). In addition, investigators found a 15% incidence of CIN in patients without CKD and 27.4% risk of CIN development in patients with CKD following PCI procedure (Mehran and Nikolsky, 2006). Prevalence of CIN increased from 8-92% as the baseline plasma creatinine levels increased from 1.5 to 6.8 mg/dL, (McCullough *et al.*, 1997).

Diabetic nephropathy is associated with a decrease in NO production leading to low levels of oxygen in the outer medulla, resulting in renal ischemia (Heyman *et al*., 2005). The incidence of CIN in patients with diabetes mellitus ranges from 5.7-29.4% (Mehran & Nikolsky, 2006). In a study by Berns and co-workers, diabetes was associated with a 27% incidence of CIN when baseline plasma creatinine was between 2.0 to 4.0 mg/dL and 81% with plasma creatinine greater than 4.0 mg/dL (Berns, 1989). In another study with a total of 421 patients undergoing coronary

angiography, CIN occurred in 11.4% of pre-diabetics, 20% of diabetics and 5.5% of patients with normal fasting glucose levels (Toprak *et al.*, 2007). In this study, CIN was defined as an increase of ≥25% in baseline serum creatinine within 2 days following angiography procedure (Toprak *et al.*, 2007). Ahuja *et al*. (2000), reported that nephrotoxic drugs (cyclosporine) and a higher prevalence of diabetes mellitus and renal impairment resulted in increased incidence of CIN development. In this retrospective study, investigators reported a 21.2% CIN incidence in 144 patients with a functional renal allograft exposed to contrast media (Ahuja *et al*., 2000).

In several studies the presence of cardiac diseases such as heart failure and acute myocardial infarction positively predicted CIN development (Rich & Crecelius, 1990; Rihal *et al*., 2002; Marenzi *et al*., 2004; Mehran *et al*., 2004). Nash *et al.* reported a 49% incidence of CIN following cardiac catheterization and angioplasty particularly in patients with cardiac disease (Nash *et al.*, 2002). In a study of 208 patients with acute myocardial infarction undergoing PCI, incidence of anterior myocardial infarction was higher in patients who developed CIN, making it a risk predictor of CIN (Marenzi *et al*., 2004). Additionally, in a retrospective study by Bartholomew and colleagues, among 2.0% of PCI patients who developed CIN, there was a 15-fold increase in major cardiac events and a 5.5-fold increase in myocardial infarction of patients who developed CIN compared to patients without CIN (Bartholomew *et al*., 2004).

1.4.2. Procedure level CIN risk factors

1.4.2.1. Contrast volume and osmolarity of contrast media

The volume of contrast medium administered is concidered the main modifiable risk factor for CIN. Contrast induced nephropathy is associated with higher levels of contrast media volume which cause increased renal function failure in the presence of other risk factors (Mehran & Nikolsky, 2006). The CIN Concensus Working Panel concluded that for every 100 ml increase in contrast media, there is a 12% increase in the risk of CIN development (Rihal *et al*., 2002; Davidson *et al.*, 2006). Additionally, the risk of CIN was lower in patients who were receiving a dose of contrast media less than 100 ml (McCullough *et al*., 1997).

Contrast media substances are classified into three types: high-osmolar (HOCM), low-osmolar (LOCM), and iso-osmolar (IOCM) with osmolarity around 2000, 600 to 800, and 290 mOsm/kg H_2O, respectively (Davidson *et al.*, 2006; McCullough, 2008). However, osmolarity variations

may vary in different lots of contrast media with the same generic name as illustrated in Table 1.2 (Davidson *et al.,* 2006).

A higher risk of nephrotoxicity was caused by using a hyperosmolar contrast media compared to a low-osmolar contrast agent (Barrett & Carlisle 1993; Parfrey, 2005). Recent studies associated a decreased risk of CIN development with low-osmolar or iso-osmolar agents and suggested that an IOCM offers a lower risk of developing CIN than LOCM in patients with high-risk factors (Aspelin *et al*., 2003). However, Schwab *et al*. (1989) found no association between various types of contrast media and risk for developing CIN.

Table 1.2. Features of some contrast media

Generic name	Type (Ionicity)	Iodine content (mg/mL)	Osmolarity (mOsm/kg H_2O)	Viscosity at 37°C (mPa.s) *
Diatrizoate	HOCM (Ionic)	140-462	550-2938	1.4-19.5
Iothalamate	HOCM (Ionic)	141-480	600-2400	1.5-9.0
Ioxithalamate	HOCM (Ionic)	120-380	610	1.1-8.5
Ioxaglate	LOCM (Ionic)	160-350	295-680	1.7-10.5
Iohexol	LOCM (Non-ionic)	180-350	~ 410-780	2.2-10.6
Iopamidol	LOCM (Non-ionic)	150-370	300-832	1.5-9.5
Iopromide	LOCM (Non-ionic)	150-400	340-880	1.2-12.3
Iobitridol	LOCM (Non-ionic)	250-350	585-915	4.0-10.0
Iomeprol	LOCM (Non-ionic)	150-400	301-730	1.4-12.6
Iodixanol	IOCM (Non-ionic)	150-320	290**	1.7-11.1

* Pure solution in water. ** After adjustment for use.

Reproduced with permission from Davidson *et al*., 2006.

1.5. Association of morbidity and mortality with CIN

Contrast induced nephropathy is associated with higher morbidity and mortality rates (McCullough *et al*., 1997; Rihal *et al*., 2002). According to Pucelikova and colleagues, investigators documented a 12-14% incidence of CIN during hospitalization post iodinated contrast media administration (Pucelikova *et al*., 2008). In several observational and retrospective studies, CIN caused a 7-22% or more in-hospital mortality (Levy *et al.,* 1996; McCullough *et al*., 1997; Rihal *et al*., 2002).

In an earlier observational study of 1826 patients who underwent PCI, investigators reported that in-hospital death rates for patients without CIN were 1.1%, 7.1% for patients with CIN, and especially high rates (35.7%) in CIN patients requiring renal dialysis (McCullough *et al*., 1997). Similarly, in a study of more than 7500 patients undergoing PCI, 22% of the patients who developed AKI died in the hospital compared to 1.4% of patients without AKI (Rihal *et al*., 2002). In this study, one-year mortality rates were documented as 12.1% for patients with renal failure versus 3.7% for those without renal failure and increased to 44.6% versus 14.5% at five years (Rihal *et al*., 2002).

In a retrospective study of 16248 patients undergoing various contrast procedures such as angiography and CT scans, Levy *et al*. found that mortality rates in patients with CIN were almost 5 times higher (34%) compared to the rates observed in patients without renal failure (7%) (Levy *et al.,* 1996). Similarly, in another study of more than 20000 patients, CIN patients had a 22-fold increase in mortality rate compared with patients without acute renal failure (Bartholomew *et al*., 2004). Patients with renal failure requiring dialysis had a 27.5% death rate post-PCI compared with 1% for patients who did not require renal dialysis (Gruberg *et al*., 2001).

Dangas *et al*. reported high levels (6.3%) of mortality in patients with CKD who developed CIN compared with the lower rate (0.8%) of mortality observed among patients with CKD who did not develop CIN (Dangas *et al*., 2005). A mortality rate of 2.5% occurred in patients with CIN and was higher than the 0.1% mortality rate of patients without CIN demonstrating that CIN is associated with poor outcomes even in patients without underlying CKD (Dangas *et al*., 2005). In this study CIN was defined as an increase in baseline plasma creatinine of ≥25% or ≥0.5 mg/dL during first 48 hours (Dangas *et al*., 2005).

The one- year mortality rates were higher among patients presenting with both baseline CKD and acute renal failure (22.6%) when compared to the 6.9% observed in patients without CIN (Dangas *et al*., 2005). In a study of 2082 patients who underwent PCI for acute myocardial infarction (AMI), there was a more than 7-fold (3.2 versus 23.3%) increase in the 1-year mortality in patients who developed acute renal failure (Sadeghi *et al.,* 2003).

1.6. Candidate genes implicated in contrast induced acute kidney injury

In previous studies, candidate genes such as interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), angiotensin-converting enzyme (*ACE*), vascular endothelial growth factor (*VEGF*), and angiotensinogen (*AGT*) were implicated in AKI pathogenesis including CIN (Stafford-Smith *et al.,* 2005; Isbir *et al.,* 2007; Liu *et al.,* 2009; Cardinal-Fernández *et al.,* 2013; Ba Aqeel *et al.,* 2017). Hence, one's genetic susceptibility to a disease such as CIN is considered an important risk factor.

1.6.1. Cytokine-induced inflammation in contrast-induced AKI

Inflammation is associated with the development of AKI, and cytokines are defined as a group of soluble messenger molecules or proteins produced locally and secreted by immune cells to the site of injury or infection (Zhang & An, 2007; Ortega & Fornoni, 2010; Silver *et al*., 2015). These molecules are normally found at low levels in the tissues and circulation, sometimes below the detection limit in tissues (Zhang & An, 2007; Ortega & Fornoni, 2010). Furthermore, investigators reported that cytokines have damaging effects thus there are various systems that play a role in regulating and neutralizing pro-inflammatory cytokines (Ortega & Fornoni, 2010). These systems include the production of IL-10 in response to the release of TNFα, resulting in a decrease in the production of TNFα (Ortega & Fornoni, 2010).

In previous studies, evidence strongly implicates inflammation in the mechanism of CIN and other forms of ischaemic AKI with inflammation playing a significant role in the initiation and extension phases of AKI (Akcay *et al.,* 2009; Ortega & Fornoni, 2010; Sharfuddin & Molitoris, 2011; Deng *et al.,* 2015). Proinflammatory cytokines play an important role as predictive biomarkers of both disease and prognosis (Ortega & Fornoni, 2010). Furthermore, high levels of cytokines are associated with detrimental outcomes in patients with AKI (Akcay *et al.,* 2009). Polymorphisms in these inflammatory cytokine genes are strongly implicated in renal disease including CIN (Ortega & Fornoni, 2010). Therefore, knowledge of the key roles played by inflammatory

cytokines in CIN is critical to laying down workable interventions for CIN and in guiding directed therapies.

1.6.2. Interleukin-6 gene (*IL-6*)

Interleukin-6 is a multifunctional cytokine, regulating pathological and physiological incidents including granulocyte development, hepatocyte generation and autoimmune diseases amongst others (Croker *et al.,* 2012). It is regarded as a proinflammatory cytokine influencing an inflammatory cascade through activation of macrophages and lymphocytes (Ding *et al*., 2020). In humans, the gene encoding IL-6 is located on chromosome 7p21 and composed of five exons and four introns (Sehgal *et al.,* 1986; Yasukawa *et al.*, 1987; Bowcock *et al*., 1988). According to previous investigators, the *IL-6* polymorphism consists of a single nucleotide change from guanine (G) to cytosine (C) at position -572 in the promotor region of the gene (Zavaleta-Muñiz *et al.*, 2013; Lin *et al.*, 2014). Interleukin-6 polymorphisms in the promoter region, in particular -572 G/C (rs1800796), are associated with transcription of IL-6 gene and its secretion (Terry *et al.*, 2000). According to Saw and colleagues, the *IL-6* -572 C allele is associated with high levels of circulating IL-6 that result in an exaggerated inflammatory response (Saw *et al.,* 2019).

In some studies, *IL-6* had a positive association with acute renal insufficiency or AKI. In one study, Stafford-Smith and colleagues found that a combination of the angiotensinogen (*AGT*) gene +842C allele (rs699) and the *IL-6* –572C allele in Caucasians had a significant association with renal dysfunction (Stafford-Smith *et al.,* 2005). In addition, Gaudino *et al*. (2003) found a significant association of the *IL-6* −174G/C polymorphism with AKI. This study found that IL-6 -174 G/C polymorphism regulates postoperative IL-6 levels and is associated with renal and pulmonary failure after coronary surgery (Gaudino *et al*., 2003). Furthermore, Jouan *et al.* (2012) observed a correlation between the *IL-6* −572 G/C polymorphism and AKI post cardiac surgery. In a meta-analysis study, Ding *et al.* (2020) reported a significant association between *IL-6* −572 G/C polymorphism and myocardial infarction. This study further reported that *IL-6* gene −572 C allele may be a protective factor for myocardial infarction (Ding *et al.*, 2020).

1.6.3. Interleukin-8 gene (*IL-8*)

Interleukin-8 is a chemoattractant cytokine produced by multiple cell types such as neutrophils, macrophages, epithelial, fibroblast, and tumor cells (Bickel, 1993; de Fontnouvelle *et al.*, 2017). The release of IL-8 from these cells occurs due to an inflammatory stimulus (Matsushima &

Oppenheim, 1989). Interleukin-8 preferentially targets neutrophil cells compared with other cell types (Bickel, 1993). Thus, this chemokine functions as a mediator of inflammation assisting in neutrophil recruitment to the site of damage and degranulation (Koch *et al.,* 1992; Dobreva *et al*., 2006). The human IL-8 gene is located on chromosome 4q13-q21 consisting of a proximal promoter region, four exons, and three introns (Gerszten *et al.,* 1999). According to previous studies, an A/T SNP (rs4073) located at position -251 in the transcription initiation site of IL-8 gene is associated with IL-8 expression (Ohyauchi *et al*., 2005; Canedo *et al*., 2008; Sun *et al*., 2016).

In a prospective study of 412 children who underwent cardiopulmonary bypass, de Fontnouvelle *et al*. reported that increased levels of preoperative IL-8 were strongly associated with the development of AKI in children 2 years and older (de Fontnouvelle *et al.,* 2017). Similarly, high levels of IL-8 were linked to AKI risk in patients who underwent liver transplants, patients with acute lung injury, and those in septic shock (Liu *et al.,* 2007; De Pablo *et al.,* 2013; Sirota *et al.,* 2013; Liu *et al.,* 2014). In another study of children undergoing cardiac surgery, researchers reported that elevated levels of IL-8 in serum was associated with AKI (Tashiro *et al.,* 2002). In addition, several studies found that urinary levels of IL-8 cytokine were elevated in patients with AKI (Tashiro *et al.,* 2002; Sirota *et al*., 2013; Borst *et al*., 2015).

Increased levels of IL-8 measured in children undergoing cardiac surgery demonstrated strong associated between IL-8 and the risk of developing AKI (Liu *et al.,* 2009). In addition, de Fontnouvelle and colleagues concluded that inflammatory cytokines including IL-8 and TNFα were positively associated with higher odds of prolonged hospital stay in children 2 years and older (de Fontnouvelle *et al.,* 2017). In another study of patients with septic shock, Cardinal-Fernández and colleagues reported that *IL-8* −251 AA (rs4073) variant was significantly associated with a higher risk of AKI development, $p = 0.045$ (Cardinal-Fernández *et al*., 2013). Additionally, higher levels of IL-8 plasma were associated with the *IL-8* -251 AA genotype than the *IL-8* -251 TT genotype (Cardinal-Fernández *et al*., 2013). Similarly, in a meta-analysis study conducted by Nazir *et al*. (2014), investigators found that *IL-8* -251 AA genotype was positively associated with an increased risk of diabetic nephropathy in the Indian population. In a study among the Chinese Han population, Zhang *et al.* (2011) also reported an increased risk for acute coronary syndrome (ACS) in patients carrying polymorphism −251 AT in the *IL-8* gene ($p = 0.004$). Shanmuganathan

et al. (2017) concluded that *IL-8* +781 C/T polymorphism demonstrated a significant positive association with diabetic CKD patients and functions as an inflammatory marker for CKD.

1.6.4. Angiotensin-converting enzyme gene (*ACE*)

Angiotensin-converting enzyme (ACE) is a vasomotor regulatory gene coding for the angiotensin converting enzyme that cleaves the angiotensin I protein at a specific location, converting this protein to angiotensin II (Vilander *et al.,* 2015). Angiotensin II acts on the central nervous system resulting in vasoconstriction and an increase in aldosterone hormone release, which helps maintain normal blood pressure by retaining sodium and water (Dandona *et al.,* 2007). Thus, the *ACE* gene is associated with kidney function by regulating blood pressure (Isbir *et al.,* 2007). The *ACE* gene is located on chromosome 17q23 consisting of twenty-six exons and twenty-five introns which regulates the plasma levels of ACE in 20% to 50% of subjects, and codes for the ACE enzyme (Hubert *et al*., 1991; Isbir *et al.,* 2007). The ACE gene polymorphism in a form of insertion (I allele) and/or deletion (D allele) of 287 bp *Alu* repeat sequence (rs4646994) is present in intron 16 of the gene (Lin *et al*., 2014).

Castellon and Hamdi (2007) reported that the I/D polymorphism may affect the expression of *ACE* gene and the function of angiotensin I converting enzyme. In a previous study, the DD genotype was associated with the thickness of vessel walls and elevated blood pressure (Dietze & Henriksen, 2008). In another study, investigators reported that the presence of the D allele is associated with higher ACE enzyme activity and higher production of angiotensin II in comparison to the I allele (Rigat *et al*., 1990).

The association of the *ACE* gene with AKI remains inconclusive due to different outcomes in previous studies. These studies investigated a 287 bp *Alu* insertion/deletion (I/D) polymorphism (rs4646994) in intron 16 of the *ACE* gene (Stafford-Smith *et al.,* 2005; Isbir *et al.,* 2007; du Cheyron *et al.,* 2008; Pedroso *et al.,* 2010; Cardinal-Fernandez *et al.,* 2013; McBride *et al.,* 2013). In a Turkish study of patients undergoing cardiac surgery, Isbir and co-workers reported acute renal failure in 54 patients (21.8%) post cardiac surgery. In addition, these investigators found a significant association between the *ACE* D allele (p= 0.018) and an increased risk of AKI after cardiac surgery (Isbir *et al*., 2007).

Contradictory to this however, du Cheyron *et al.* (2013) found that the frequency of the rs4646994 II genotype was higher in patients with AKI as compared to those without (42% versus 13%).

Thus, the *ACE* II genotype is associated with the increased risk for AKI development and outcome (p= 0.0002) in a Caucasian population (du Cheyron *et al.*, 2008). The remaining studies failed to detect association between the *ACE* SNP and AKI either in the intensive care unit or after cardiac surgery (Pedroso *et al*., 2010; Cardinal-Fernández *et al*., 2013; McBride *et al*., 2013). In a previous study, investigators found no significant association between the *ACE* II genotype and AKI among the white population (Cardinal-Fernández *et al*., 2013). Similarly, in a study by McBride *et al*. (2013), the *ACE* D allele was not associated with AKI in a white population located in United Kingdom. In addition, Pedroso *et al.* (2010) found no association between genotypic or allelic frequencies of the *ACE* SNP and AKI among the southern Brazilian population.

1.6.5. Vascular endothelial growth factor gene (*VEGF*)

Vascular endothelial growth factor is a potent angiogenesis factor involved in kidney development and renal repair (Maeshima & Makino, 2010). Vascular endothelial growth factor stimulates endothelial cell survival, proliferation and differentiation and maintains a healthy vasculature (Reinders *et al*., 2003; Schrijvers *et al.*, 2004; Doi *et al.*, 2010). Furthermore, this protein functions as a proinflammatory cytokine by increasing vascular permeability (Neufeld *et al.*, 1999; Ferrara & Gerber, 2001; Reinders *et al*., 2003).

According to Vincenti and colleagues, the human VEGF gene is located on chromosome 6p21.3 and is organised into eight exons and seven introns. Vascular endothelial growth factor gene has at least 30 functional SNPs in the 5′-untranslated region (UTR), 3′- UTR, and promoter regions (Vincenti *et al.*, 1996). The main focus has been on SNPs found in regulatory regions because they may alter VEGF expression levels (Liu *et al*., 2018). The *VEGF* +405 G/C polymorphism (rs2010963) is located within the 5′- UTR and may affect transcription factor binding affinity (Watson *et al.*, 2000; Stevens *et al.*, 2003). Furthermore, Henidi *et al*. (2015) stated that the functional polymorphisms including *VEGF* +936 C/T SNP (rs3025039) located in exon eight, corresponding to the 3′- UTR of the gene were reported to be associated with differential VEGF expression. In a previous study, researchers reported that +936 C/T (rs3025039) and +405 G/C (rs2010963) among other SNPs play a pivotal role in VEGF protein synthesis (Maeda *et al.*, 2013). The C allele of *VEGF* +936C/T SNP is associated with substantially increased serum VEGF levels whereas the +936 T allele is correlated to lower VEGF expression levels (Renner *et al.*, 2000; Krippl *et al.*, 2003).

In a prospective cohort study of 139 patients diagnosed with severe sepsis, investigators reported that the *VEGF* +936 (rs3025039) CC-genotype was associated with increased risk of AKI (Cardinal-Fernández *et al.,* 2013). In another study of 101 male patients treated with haemodialysis, investigators reported a strong association between the 936C/T and 1451C/T polymorphisms found in the 3' UTR of the *VEGF* gene and end stage renal disease (ESRD) (Doi *et al*., 2006). In a study of 211 Swedish patients undergoing dialysis, levels of VEGF did not differ between patients with CKD 5 and those without (controls in this study) following dialysis treatment (Yuan *et al*., 2013). However, circulating high levels of VEGF were associated with inflammation biomarkers and regarded as a predictor of mortality (Yuan *et al*., 2013).

In previous studies, investigators studied the association of *VEGF* (rs2010963 and rs3025039) polymorphisms with diabetic retinopathy, breast cancer and coronary artery disease (Fan *et al.,* 2014; Rezaei *et al.,* 2016; Ma *et al.,* 2018). In a cross-sectional study conducted by Fan and colleagues, no evidence of association was observed between +405C/G (rs2010963), +936C/T (rs3025039) variants and diabetic retinopathy risk in a Chinese population (Fan *et al.,* 2014). Similarly, Rezaei *et al.* (2016) found that the genotypes and allele frequencies of rs3025039 (+936C>T) and rs2010963 (+405C>G) polymorphisms were not associated with risk/protection of breast cancer in an Iranian population. In contrast, a meta-analysis study by Ma and co-workers indicated that the *VEGF* gene polymorphisms of rs2010963 (+405C/G) and rs3025039 (+ 936C/T) were associated with an increased risk of coronary artery disease (Ma *et al.,* 2018).

1.6.6. Angiotensinogen gene (*AGT*)

Angiotensinogen is a protein produced in the liver found circulating in plasma. It functions as a precursor of angiotensin (Ang) peptides and forms part of the non-inhibitory serpin superfamily (Lu *et al.,* 2016; Ba Aqeel *et al.,* 2017). Angiotensin II (Ang II), produced by the renin-angiotensin system (RAS) which regulates blood pressure in the body, induces vasoconstriction and a rise in blood pressure (Lu *et al.,* 2016; Satou *et al.,* 2018). In addition, Satou and co-workers reported that an increase in Ang II results in several pathophysiological mechanisms including production of ROS and alterations in renal haemodynamic, mechanisms associated with CIN (Satou *et al.,* 2018).

The human AGT gene is located on the long arm of chromosome 1 (1q42-q43) and consists of five exons and four introns (Moon *et al.,* 2013; Merlo *et al.,* 2016). The AGT polymorphism, rs699 (also referred to as M235T) is a thymine (T) to cytosine (C) substitution in the exon two which

resulted in a functional methionine (M) to threonine (T) amino acid exchange at codon 268, referred to as M268T (Makuc *et al.*, 2017). The AGT polymorphism (rs699) is referred to as M235T because it was previously positioned to the amino acid 235 (Makuc *et al.,* 2017). Previous studies reported that the rs699 (M235T) polymorphism is associated with plasma AGT concentrations and the elevated levels of AGT are seen in the TT homozygous genotypes (Jeunemaitre *et al.*, 1992; Jeunemaitre *et al.*, 1992; Bloem *et al.*, 1997). In addition, Makuc and colleagues stated that the rs699 threonine variant is associated with higher plasma AGT levels and higher blood pressure (Makuc *et al.,* 2017).

Several studies have reported that AGT is positively associated with AKI (Alge *et al*., 2013; Alge & Arthur, 2015). Ba Aqeel and colleagues described AGT as a biomarker of RAS overactivity in kidney diseases and hypertension (Ba Aqeel *et al.*, 2017). Angiotensinogen as a biomarker has prognostic importance which suggests a role for RAS activation in regulating the severity of AKI and its outcomes (Ba Aqeel *et al*., 2017). In a study of 99 urine samples from patients who had cardiac surgery, increased levels of the urine angiotensinogen/creatinine ratio (uAnCR) were linked with AKI causing adverse outcomes (Alge *et al*., 2013). Angiotensinogen was defined as a prognostic biomarker of AKI following cardiac surgery (Alge *et al*., 2013; Alge & Arthur, 2015). In addition, investigators found that a combination of *AGT* +842 T/C polymorphism and *IL-6*-572C allele in Caucasians is associated with renal dysfunction (Stafford-Smith *et al.,* 2005). Although AGT gene +842C allele (rs699) is considered as a prognostic biomarker, its use as a diagnostic biomarker is limited (Kashani *et al.,* 2017). Therefore, further validation is required in other clinical settings.

In summary, genetic susceptibility to CIN is associated with polymorphisms in the *ACE*, *IL-6*, *IL-8*, *VEGF*, and *AGT* genes although these associations have not been studied in South African patients. Identification of these polymorphisms is critical in implementing targeted modalities that will lessen the impact of CIN among patients. Our study therefore is to investigate an association of single nucleotide polymorphisms (SNPs) in the *ACE*, *IL-6*, *IL-8*, *VEGF*, and *AGT* genes on CIN in a black South African population undergoing contrast media administration.

1.7. RATIONALE OF THE STUDY

In developed countries, CIN ranks third as the cause of hospital acquired AKI, impacting highly on morbidity and mortality (Chang *et al.*, 2013). However, in sub-Saharan Africa, information on prevalence and risk factors for CIN including patient outcomes remained unexplored until a study by Banda *et al.*, 2016. Banda *et al.* (2016) showed that CIN has a relatively high incidence (the rate of CIN was 16.4%) and that it positively predicted mortality in patients receiving contrast media (Figure 1.2). Despite identification of traditional risk factors for CIN and its pathogenesis from studies in developed countries, information on the contribution of susceptibility genes to the development of CIN and its severity amongst African populations is lacking.

Before the study by Banda (2017), no studies had investigated susceptibility genes for CIN in black African populations. In his study, Banda looked at the influence of polymorphisms in the *TNFα* and *IL-10* genes on CIN and found that *TNFα* and *IL-10* genetic polymorphisms had a positive association with risk of CIN and CIN mortality (Banda, 2017). However, the influence of genetic polymorphisms in the *ACE, IL-6, IL-8, VEGF* and *AGT* genes remains unexplored in CIN patients from South Africa.

Therefore, this study set out to identify if SNPs associated with susceptibility to CIN in other populations were also associated with CIN amongst black South Africans. Identification of susceptibility genes will not only contribute to reducing morbidity and mortality but will also add knowledge to renal medicine in this population.

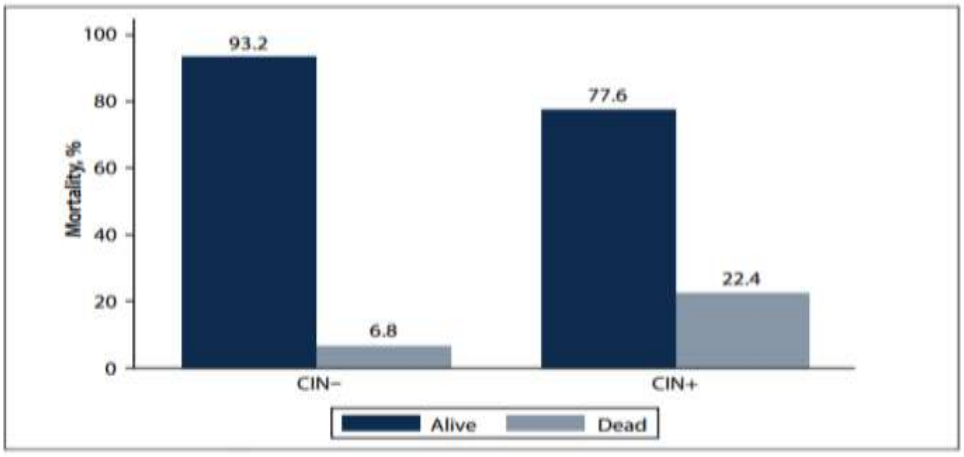


Figure 1.2: Contrast-induced nephropathy and mortality (CIN- v. CIN+, p<0.001).

Taken from Banda *et al*., 2016.

1.8. RESEARCH HYPOTHESIS

In the present study we hypothesized that single nucleotide polymorphisms of the *ACE* (rs4646994), *IL-6* (rs1800796), *IL-8* (rs4073), *VEGF* (rs3025039 and rs2010963) and *AGT* (rs699) genes are associated with an increased risk of CIN in a black South African population.

1.9. RESEARCH AIM

The aim of this study was to determine genetic susceptibility for Acute Kidney Injury (AKI) in patients receiving contrast media.

1.10. STUDY OBJECTIVES

The main objectives for this study are as follows:

1. Extract DNA from peripheral blood samples collected from patients that have undergone contrast media administration.
2. Determine genotypes for SNPs located in the *IL-6* (rs1800796), *IL-8* (rs4073), *VEGF* (rs3025039 and rs2010963) and *AGT* (rs699) genes using TaqMan® SNP genotyping assays.
   To genotype the ACE (rs4646994) SNP using end-point PCR followed by electrophoresis on a 1% agarose gel.
3. Determine association of SNPs located in the *ACE* (rs4646994), *IL-6* (rs1800796), *IL-8* (rs4073), *VEGF* (rs3025039 and rs2010963) and *AGT* (rs699) genes with contrast induced AKI.

Chapter 2

2. SUBJECTS AND MATERIALS

2.1. Ethics clearance

This study was approved by the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (certificate number. M190692-Appendix A). Ethical approval for the parent study was also obtained from the HREC of the University of the Witwatersrand (certificate number. M190762-Appendix B). Permission to use whole blood samples was granted by Dr Justor Banda (Appendix C).

2.2. Study design, setting and population

This retrospective case-controlled study forms part of a larger study entitled "Biomarkers in acute kidney injury due to contrast induced nephropathy" that was conducted at the Charlotte Maxeke Johannesburg Academic Hospital in South Africa. The parent study included patients undergoing computerized tomography (CT) and angiography in the Divisions of Radiology and Cardiology from July 1, 2014 to July 30, 2015. Iopamidol (Jopamiron) a low osmolar contrast agent available in 200 to 370 mg/ml was used in all CT scan and angiography procedures. Patients were recruited and followed-up for the development of CIN. Patients recruited to the study who did not develop CIN were designated as controls.

A subset of patients from the parent study comprising 228 black African patients, 30 of whom developed CIN and 198 of whom were controls, were included in this genetic study and genotyped for polymorphisms in the *ACE*, *IL-6*, *IL-8*, *VEGF* and *AGT* genes.

2.2.1. Inclusion criteria

For the study, consenting patients aged 18 years or older, undergoing computed tomography contrast media administration or angiography and included patients with stable chronic kidney disease (CKD). Contrast media administration was given to all patients in the study.

2.2.2. Exclusion criteria

Patients with any of the following conditions were excluded: Participants below 18 years, evidence of pre-existing AKI (clinical or laboratory), end stage renal disease (ESRD) on renal replacement therapy (RRT), prior contrast media administration in the preceding 7 days, pregnancy, and non-consenting participants.

2.2.3. Study outcomes

The primary endpoint of the parent study was the occurrence of CIN, defined as a serum creatinine increase >25% from baseline or at least 44 µmol/l assessed within 48-72 hours post contrast media administration as per the KDIGO definition. Secondary endpoints were mortality and identification of CIN susceptibility genes to add knowledge to renal medicine.

2.2.4. Samples

A 5 ml sample of whole blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) to prevent clotting and stored at -20 °C for genomic DNA extraction.

2.3. Genomic DNA extraction and genotyping

2.3.1. DNA extraction

- **Extraction with the Automated platform**

Genomic DNA was extracted from whole blood samples collected in EDTA tubes, using the automated Maxwell® 16 DNA Purification Kit from Promega (Madison, Wisconsin, USA), according to the manufacturer's instruction. Appendix F describes the protocol.

- **Extraction with salting out method**

For the samples in which insufficient DNA was extracted with the automated platform, a standard salting out method was used (modified from Miller *et al*., 1988, Appendix G).

In brief, for the salting out method, lysis buffer was added to the whole blood samples to break down the cell membranes of the blood cells. The cell lysates were digested overnight with a 10% SDS and Proteinase K solution (day 1). The following day (day 2), saturated NaCl was added to the mixture and the samples centrifuged to precipitate and remove the protein. The pellets were discarded, and ethanol was added to the supernatant to precipitate the DNA. If the DNA was visible, it was removed with a pipette tip, washed twice in 70% ethanol to remove excess salt, and resuspended in 100 µl of TE buffer. The samples were left overnight at a temperature of 37 °C to ensure that the extracted DNA dissolved completely before undergoing quantification.

For samples that did not aggregate (DNA was not visible), the tubes were placed in a -20 °C freezer overnight. The samples were taken out of the freezer on day 3, centrifuged and the supernatant discarded. The pellets in the tubes were washed twice in 70% ethanol, left to air dry and

resuspended in 100 µl of TE buffer overnight on the mini shaker (Biosan, Riga, Latvia) at room temperature to allow the pellets to dissolve. The resuspended DNA samples were stored at -20 °C until required (Full protocol in Appendix G).

2.3.2. DNA quantification

The concentration and quality of the DNA samples was examined on the NanoDrop™ 2000 spectrophotometer (Thermo-Fisher Scientific, Wilmington, Delaware, USA). The following were recorded for each sample; DNA concentration (ng/µl), absorbance at 260 and 280 nm wavelengths, and the A260/280 ratio. The DNA protein ratio, 260/280, is used as an indicator of DNA purity. A ratio of ~1.8 indicates pure DNA, a result less than one is suggestive of significant protein contamination (Mackey & Chomczynski, 1997).

2.3.3. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a scientific technique in molecular biology that allows selective exponential amplification of a specific (targeted) DNA sequence. Each PCR reaction involves several cycles of varying temperatures that result in DNA denaturation, primer annealing and primer extension and hence DNA replication (Mueller and Young, 2003). There are two types of PCR: Conventional and Real-Time PCR. With conventional PCR, completed PCR reactions containing amplified DNA fragments are analysed and viewed by gel electrophoresis. In contrast, Real-Time PCR permits the analysis of the products while the reaction is in progress by using various fluorescent dyes that react with amplified products and are measured by an instrument (Joshi & Deshpande, 2011).

2.3.3.1. Conventional PCR for the *ACE* SNP (rs4646994)

The extracted genomic DNA was used as a template to amplify a region of intron 16 of the *ACE* gene. In a previous study, the *Alu* insertion (I) in intron 16 of the *ACE* gene resulted in premature codon termination, so the *Alu* I has only one active site in the N-domain and the *Alu* deletion allele (D) still has two active sites (Widodo *et al.,* 2017). Angiotensin-converting enzyme polymorphism (*ACE* SNP) was detected by performing two separate PCR reactions (labeled as PCR1 and PCR2). PCR1 detects both the I and D alleles (599 and 312 bp amplicons, respectively). Because the D allele was preferably amplified and has two active sites, DD genotypes were subject to a second PCR (PCR2) which detects the I allele (335 bp amplicon) by using insertion specific primers, preventing ID/DD mistyping.

- **Polymerase chain reaction 1 (PCR1)**

The PCR1 reaction was set up using forward primer 5'-GCC CTG CAG GTG TCT GCA GCA TGT-3' (*ACE*_F) and reverse primer 5'-GGA TGG CTC TCC CCG CCT TGT CTC-3'(*ACE*_R) (Nakazawa *et al.,* 2012). The PCR reactions consisted of water, 5X MyTaq reaction buffer (comprises of 5 mM dNTPs, 15 $MgCl_2$, stabilizers and enhancers) (Bioline, Luckenwalden, Germany), forward and reverse primers (IDT, Coralville, Iowa, USA), MyTaq DNA polymerase (Bioline, Luckenwalden, Germany) and DNA template. Reaction conditions are as shown in Table 2.1. The reactions were amplified on the MJ Mini^{TM} Thermal cycler (Bio-Rad, Foster City, California, USA). The cycling conditions were as follows: an initial denaturation step at 95 °C for 1 minute, followed by 35 cycles with denaturation at 95 °C for 15 seconds, annealing at 60 °C for 15 seconds, extension at 72 °C for 10 seconds and a final extended extension cycle of 72 °C for 1 minute followed by holding temperature at 4 °C. Amplification of the D allele was characterised by a 312 bp fragment, whereas a 599 bp fragment indicated the I allele (Widodo *et al.,* 2017). Amplified products were analysed by agarose gel electrophoresis.

- **Polymerase chain reaction 2 (PCR2)**

Each sample genotyped as DD, was subjected to the second PCR to amplify a region interior to the intron 16 insertion with forward and reverse primers (IDT, Coralville, Iowa, USA) specific for the *Alu* repeat sequence 5'-TGG GAC CAC AGC GCC CGC CAC TAC-3' (*ACE*_II_F) and 5'-TCG CCA GCC CTC CCA TGC CCA TAA-3' (*ACE*_II_R) to avoid ID-DD mistyping (Nakazawa *et al.,* 2012). Reaction setup for the second PCR is shown in Table 2.2 and the cycling conditions are the same as for PCR1. Amplification of the I allele in PCR2 was characterised by a 335 bp amplicon size (Nakazawa *et al.,* 2012; Widodo *et al.,* 2017). The PCR products were analysed on a 1% agarose gel by electrophoresis.

For quality control, negative controls (water-blanks) were included in both PCR reaction setups to identify any possible contamination. The negative control did not contain DNA template but contained all other reagents and was subjected to the same amplification procedures as the samples.

Table 2.1 Reagents, volumes and concentrations used to prepare PCR1 products

Reagents	Concentrations	Volume (µl)	Manufacturer
Water	-	11.4	Sabax
MyTaq Buffer	5X	4	Bioline
*ACE*_F	10 µM	1	IDT
*ACE*_R	10 µM	1	IDT
MyTaq DNA Polymerase	5 U/µl	0.1	Bioline
DNA	10 ng/µl	2.5	-
Reaction Volume		**20 µl**	

This template is for a single sample and was scaled up for the total number of samples in the experiment.

Table 2.2 Reagents, volumes and concentrations used to prepare PCR2 products using insertion-specific primers

Reagents	Concentrations	Volume (µl)	Manufacturer
Water	-	11.4	Sabax
MyTaq Buffer	5X	4	Bioline
*ACE*_II_F	10 µM	1	IDT
*ACE*_II_R	10 µM	1	IDT
My Taq DNA Polymerase	5 U/µl	0.1	Bioline
DNA	10 ng/µl	2.5	-
Reaction Volume		**20 µl**	

A template for single sample used to calculate the reaction volumes. In our calculations we scaled-up master mix accordingly.

2.3.4. Agarose gel electrophoresis

Electrophoresis is a technique by which charged DNA molecules migrate through the pores of the gel under the influence of an electric current. The rate of migration is dependent on the size of the molecules and their electric charge, the negatively charged DNA molecules move from the negative to the positive electrode. The larger fragments will move at a slower rate compared to the smaller fragments thus separating fragments by size (Madigan *et al*., 2000).

Amplified PCR products were resolved on a 1 % agarose gel by electrophoresis. Briefly, 1.2 g of agarose powder (Lonza, Rockland, ME) was dissolved in 120 ml of 1X TBE (Tris-Boric Acid-EDTA, Appendix H) buffer to make up a 1% agarose solution. The mixture was heated until completely dissolved and the cylinder was placed in cold water to cool down to a temperature of about 50 – 60 °C before adding 12 µl of ethidium bromide with a concentration of 10 mg/mL (Biobasic inc, Markham, Ontario). Ethidium bromide was added to allow for visualization of the amplified PCR products under UV-light. The gel solution was cast on gel trays and left to set for 45 – 60 minutes. The amplicon (7.5 µl) was mixed with 2.5 µl of the DNA loading dye (Bioline, London, UK) and added onto the gel. Five microliters of a Generuler 100 bp DNA ladder (Genedirex, Taoyuan, Taiwan) was added to the first well of the gel to ensure that the PCR product visualized was of the correct fragment length. Samples were run at 100 volts for 60 minutes.

2.3.5. Gel imaging

Gel Doc™ EZ imager (Bio-Rad, Foster City, California, USA) was used to visualize the PCR products under UV-light. Image lab software version 6.1 (Bio-Rad, Foster City, California, USA) was used to capture and analyse digital image data from electrophoresis gels.

2.3.6. Quantitative real-time PCR

Single nucleotide polymorphisms (SNP) in the *IL-6* (rs1800796), *IL-8* (rs4073), *VEGF* (rs3025039 and rs2010963), and *AGT* (rs699) genes were genotyped using TaqMan® SNP genotyping assays (Thermo-Fisher Scientific, Waltham, USA). The context sequences for the SNP genotyping assays are shown in Table 2.3.

To check whether the alleles or variants of the genes are present in sub-Saharan African populations, the 1000 genomes database (www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) was used to check the minor allele frequencies (MAF) of each SNP in the ESN Esan (Nigeria), LWK Luhya (Kenya) and YRI Yoruba (Nigeria) populations. In addition, minor allele frequencies

were determined for the Sowetan population using publicly available data from 94 Bantu-speaking South Africans, resident in urban Soweto (May *et al.*, 2013) and analysed using Plink software (http://pngu.mgh.harvard.edu/purcell/plink/). Minor allele frequencies are shown in Table 2.4.

Table 2.3 Context Sequences for TaqMan® labelled SNP genotyping assays. The first allele of the SNP is labelled with FAM and the second allele with VIC

Gene name	**Location:**	**Context Sequence**
***IL-6* (rs1800796)**	Chr.7:22726627 on Build GRCh38	ATGGCCAGGCAGTTCTACAACAGCC**[C/G]**CTCACAGGGAGAGCCAGAACACAGA
***IL-8* (rs4073)**	Chr.4:73740307 on Build GRCh38	TTATCTAGAAATAAAAAAGCATACA**[A/T]**TTGATAATTCACCAAATTGTGGAGC
***VEGFA* (rs3025039)**	Chr.6:43784799 on Build GRCh38	GCATTCCCGGGCGGGTGACCCAGCA**[C/T]**GGTCCCTCTTGGAATTGGATTCGCC
***VEGF* (rs2010963)**	Chr.6:43770613 on Build GRCh38	CGCGCGGGCGTGCGAGCAGCGAAAG[**C**/**G**]GACAGGGGCAAAGTGAGTGACCTG
***AGT* (rs699)**	Chr.1:230710048 on Build GRCh38	CAGGGTGCTGTCCACACTGGCTCCC**[A/G]**TCAGGGAGCAGCCAGTCTTCCATCC

Table 2.4 Minor Allele Frequencies in varying African populations

GENES	Population diversity			
	Esan (Nigeria)	Luhya (Kenya)	Yoruba (Nigeria)	Soweto
IL-6 (rs1800796)	C 0.0707	C 0.2121	C 0.0787	C 0.0479
IL-8 (rs4073)	T 0.1465	T 0.1818	T 0.1435	T 0.1848
VEGF (rs3025039)	T 0.0657	T 0.0707	T 0.0694	T 0.0426
VEGF (rs2010963)	C 0.3131	C 0.3182	C 0.3056	C 0.03457
AGT (rs699)	A 0.0657	A 0.1111	A 0.0787	A 0.0426

Minor allele frequency (MAF) data for the Esan, Luhya, and Yoruba populations were retrieved from the 1000 genomes database (www.ncbi.nlm.nih.gov/variation/tools/1000genomes/, accessed May 2019). The MAF data for the Soweto population was retrieved from May *et al.*, 2013 and calculated using Plink Software (http://pngu.mgh.harvard.edu/purcell/plink/, accessed May 2019).

2.3.6.1. PCR reaction set up and amplification

TaqMan® SNP genotyping assays were carried out according to the manufacturers' protocol. In each assay, the final 10 µl reaction mixture consisted of 2 µl of water; 5 µl of the 2X Sensifast probe No-ROX master mix comprising of dNTPs, stabilizers, and enhancers (Bioline, Luckenwalden, Germany); 0.5 µl of the TaqMan primer/probe mix (20X) (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) each; and 2.5 µl of 10 ng/µl DNA (Table 2.5). The master mix and DNA samples were pipetted into a 96-well Piko PCR plate (Thermo-Fisher Scientific, Loughborough, UK). Plates also contained negative or NTC (no-template control) wells to identify any possible contamination. No-template controls did not contain DNA template hence fluorescence levels were due to the uncleaved probe. Plates were covered with a clear optical sealing film (Thermo-Fisher Scientific, Loughborough, UK) and centrifuged at 1 500 rpm for 2 minutes in an LMC- 3000 centrifuge (Biosan, Riga, Latvia) to collect the reagents at the bottom

of the wells and remove air bubbles. The plates were loaded into the PikoReal 96 Real-Time Thermal Cycler (Thermo-Fisher Scientific, Helsinki, Finland) and the assay run using a allelic discrimination protocol. Thermocycling conditions for allelic discrimination included an initial step for 30 seconds at 25 ˚C, a denaturing step for 5 minutes at 95 ℃, followed by 40 cycles, each consisting of a denaturation (95 ˚C for 5 minutes) and annealing (60 ˚C for 50 seconds) step and a final step for 10 seconds at 25 ˚C.

Table 2.5 Real-time PCR reaction setup illustrating reagents, volumes and concentrations used to prepare PCR products

Reagents	**Concentrations**	**Volume (µl)**	**Manufacturer**
Water	-	2	Sabax
Sensifast master mix	X2	5	Bioline
TaqMan primer/probe	20X	0.5	Thermo-Fisher Scientific
DNA sample	10 ng/µl	2.5	-
Reaction Volume		**10 µl**	

This template is for a single sample and was scaled up for the total number of samples in the experiment.

2.3.6.2. PikoReal software allelic discrimination (AD) analysis

Results were analysed using the allelic discrimination (AD) plots generated by the PikoReal-Real-Time PCR System software version 2.2 (Thermo-Fisher Scientific, Finland). The AD assay is a multiplexed, robust, and reproducible assay consisting of two differently labeled probes targeting the alleles (an ancestral allele and a minor allele). One of the probes was designed to be a 100% match to the first allele (ancestral), and the other probe was designed to be a 100% match to the second allele (minor). Sample IDs including six water blank samples (negative controls) were entered in the template on the computer with a specified reaction volume of 10 µl per well.

The PikoReal-Real-Time PCR System software version 2.2 measured the intensity of the fluorescent signal and samples were classified using the RFU (relative fluorescent units) method.

The RFU method is a traditional endpoint method where measured fluorescent signals of the two probes are plotted against each other for each sample. The probe of each allele is labelled with a different fluorophore (FAM and VIC) producing fluorescence at two different wavelengths, FAM on the x-axis and VIC on the y-axis (Figure 2.1). The plotted fluorescent signals were used to call genotypes for each sample. An increase in fluorescent signal of either FAM or VIC indicated a homozygote for the FAM-specific or VIC-specific allele while an increase in fluorescence in both indicated heterozygous samples with both the VIC and FAM labelled alleles (Thermo-Fisher Scientific, 2013, 80).

Table 2.6 shows alleles representing FAM (allele 1 on the x-axis) and VIC (allele 2 on the y-axis) for each SNP. Genotypes were identified based on fluorescence clustering patterns in a bivariate plot. Figure 2.1 represents an AD graph used to illustrate genotype results of the SNPs. Samples that fell outside the clusters or that were close to the no-template controls (lower left population) were repeated.

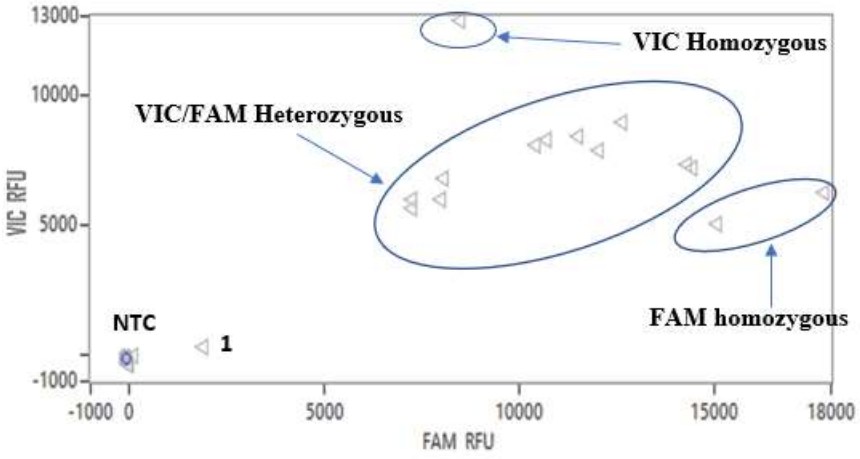


Figure 2.1: TaqMan allelic discrimination assay demonstrating the separation of different genotype populations and NTC- No template controls (negative controls). Sample labelled 1- failed to amplify. FAM (x-axis) and VIC (y-axis) represents specific alleles shown in Table 2.6.

Table 2.6 Showing SNP alleles labelled with FAM and VIC Fluorophores in the TaqMan genotyping assays

SNP ID	FAM	VIC	Ancestral allele	Minor allele
IL-6 (rs1800796)	G	C	G	C
IL-8 (rs4073)	T	A	A	T
VEGFA (rs3025039)	T	C	C	T
VEGF (rs2010963)	G	C	G	C
AGT (rs699)	G	A	G	A

2.4. Sager sequencing

2.4.1. Primer design

Target sequences of the genes of interest (*IL-6*, *IL-8*, *VEGF*, and *AGT*) were found using the National Center for Biotechnology Information (NCBI) webpage: http://www.ncbi.nlm.nih.gov/. Homo sapiens reference sequences (RefSeq) for the genes of interest were selected in the FASTA format and the context sequences (Table 2.3) used to find the target region containing the SNP of interest within the RefSeq. Sequences of at least 200 bases (the minimum amplicon size) which included the target region were inserted into the PrimerQuest tool (https://eu.idtdna.com/Primerquest/Home/Index) accessed via Integrated DNA Technologies (IDT). The general PCR design parameters were selected, and the designed primer pairs generated checked for suitability. The primer sequences are shown in Table 2.7.

To choose the correct primers, the following criteria was used:

- Total amplicon length should be 200-400 bp
- The length of the primer to be 18-24 bases
- GC content of 50% - 60% (was optimal)
- The melting temperature (Tm) of 50 – 60 °C
- Primer pairs should have a Tm within 5 °C of each other
- Primer pairs should not have complementary regions
- Avoid secondary structure formation by adjusting primer locations so they are located outside secondary structure in the target sequence, if required
- The SNP of interest should not be too close to either the 3’ or 5’ end of the amplicon

MWG Operon Eurofins program (https://www.eurofinsgenomics.eu/) was used to verify the suitability of the primers chosen for PCR.

Table 2.7 Primer Sequences for TaqMan® SNP genotyping assays

Gene	**Primer Sequences**	**Annealing Temperature**	**Amplicon Lengths**
IL-6 (rs1800796)	*IL-6*_F: 5'-CAG TGA AAC AGT GGT GAA GAG A-3'	60 °C	455 bp
	*IL-6*_R: 5'-GTG ACC AGA TTA ACA GGC TAG AA-3'	60 °C	
IL-8 (rs4073)	*IL-8*_F: 5'-TCT AAC ACC TGC CAC TCT AGT A-3'	60 °C	365 bp
	*IL-8*_R: 5'-GTG CTC TGC TGT CTC TGA AA-3'	60 °C	
VEGF (rs3025039)	*VEGFA*_F: 5'-CTC TCA CCA GGA AAG ACT GAT AC-3'	60 °C	451 bp
	*VEGFA*_R: 5'-CAA GTC CAC AGC AGT CAA ATA C-3'	60 °C	
VEGF (rs2010963)	*VEGF*_F: 5'-GAG AAG TCG AGG AAG AGA GAG A-3'	60 °C	341 bp
	*VEGF*_R: 5'-GAA CAG CCC AGA AGT TGG A-3'	60 °C	
AGT (rs699)	*AGT*_F: 5'-TCC TTG GAA GGA CAA GAA CTG-3'	60 °C	359 bp
	*AGT*_R: 5'-AGT GGA CGT AGG TGT TGA AAG-3'	60 °C	

2.4.2. DNA sequencing

Sequencing was performed to confirm the genotype results observed through allelic discrimination graphs. The sequencing results were visualized through electropherograms that are depicted in colour with each nucleotide base represented by a different colour.

For each SNP (rs1800796, rs4073, rs3025039, and rs699), one sample representative of each genotype found was sequenced to confirm AD graph results. For the rs2010963 SNP, two samples (one representing CC genotype and the other GC genotype) were sequenced to confirm the genotype results. Conventional PCR was performed on the samples to produce an amplicon that could be sent for sequencing. The PCR reaction was set up using the forward and reverse primers (IDT, Coralville, Iowa, USA) presented in Table 2.7 for each SNP. The reaction conditions were the same as shown in Table 2.1 with the only exception being the use of various forward and reverse primers as depicted in Table 2.7. The reactions were amplified on the MJ Mini^{TM} Thermal cycler (Bio-Rad, Foster City, California, USA) and the cycling conditions were the same as for PCR1 (section 2.3.3.1).

The PCR products were analysed on a 1% agarose gel to confirm amplification of samples before sending them for sequencing. A negative control (no DNA reaction) was added to identify possible contamination. For each SNP, 15 µl PCR amplicons and 10 µl forward primers at a concentration of 10 µM were sent for sequencing. Only a forward sequence reaction was done for every sample. Sequencing reactions, purification, and injections were carried out using the ABI 3500XL Genetic Analyzer performed by Inqaba biotec (www.inqababiotec.co.za).

2.4.3. Sequencing analysis

Sequences were visualised using Chromas version 2.6.6 software (www.technelysium.com.au). The results were presented as electropherograms (chromatographs). The chromatographs were analysed individually at the SNP position (rs1800796, rs4073, rs3025039, rs2010963, and rs699) to confirm the genotyping results obtained from the AD graphs for the SNPs.

2.5. Statistical analysis

2.5.1. Data management

The data were recorded and managed using a spreadsheet facilitated by the Microsoft Excel software. Genotypes at each locus were scored in all individuals and their frequencies were computed using direct gene counting.

2.5.2. Data analysis

The genotype data of each SNP from this study was statistically analysed to determine whether any significant results were obtained. Once the genotyping data for each SNP was generated, genotype and allele frequencies were calculated by gene counting. The Pearson chi-square test (χ^2 test of independence) was performed using Microsoft excel and the results were confirmed using STATA version 14.2 (College Station, Texas). *P*- values were calculated for all the tests. A *p*-value smaller than 0.05 indicated a significant effect. For the regression analysis, a β-coefficient was calculated with a value between 0 (indicates no relationship) and 1 or -1 (representing the maximum-association). Shapiro-Wilk test was used to check for normality. Continuous variables were found to be non-normally distributed and were therefore represented as medians and inter-quartile ranges (IQRs), and non-parametric, p-values were calculated using a Wilcoxon–Mann–Whitney test.

Allele and genotype frequencies were calculated for each SNP (rs4646994, rs1800796, rs4073, rs3025039, rs2010963, and rs699). Case (CIN) and control (non-CIN) frequencies were calculated separately, and the categorical data was represented as frequencies with percentages. Comparisons between patients who developed CIN and those negative for CIN were assessed using a χ^2 test of independence or a Fisher's Exact test (when any of the groups had a frequency of less than 5) depending on frequencies for categorical variables and a Wilcoxon–Mann–Whitney test for the continuous variables. Each test was performed using the four genetic modes of association (dominant, recessive, multiplicative and additive) to find out whether there could be an association using any of these genetic models. For the additive model, an association coefficient (β-Coef) and 95% confidence interval (95% CI) was calculated for the risk of developing CIN in a univariate analysis for each of the exploratory variables using statistical regression analysis. Genotypes were assessed for deviation from the Hardy-Weinberg equilibrium (HWE) using Microsoft excel.

2.6. Power calculations

Power calculations were done using an online computer program Quanto version 1.2 (http://hydra.usc.edu/gxe) developed by Gauderman and Morrison. Quanto is defined as a computer program used to calculate power or study size required for association studies of genes, environmental factors, gene to environment interaction, and gene to gene interaction (Gauderman & Morrison, 2006). This current study was designed on power calculations only.

2.6.1. Power study design

In this study, power for "unmatched case-control" studies of each SNP (*IL-6*, *IL-8*, *ACE*, *VEGFA*, *VEGF*, and *AGT*) was calculated under various assumptions including, dominant genetic model, allele frequency using MAF values of the Soweto population group (Table 2.4.), a disease prevalence of 10%, case-control ratio of 1:6, sample size referring to number of cases (30 patients), and a type 1 error rate of 0.05 (5%), which was used to further confirm association of SNPs studied with CIN. The statistical power of the study was 0.05 (5%), which indicates that this study was underpowered to detect associations of the SNPs with CIN.

Chapter 3

3. RESULTS

3.1. Demographic characteristics

In this retrospective cohort study, a total of 228 black South African participants were recruited and genotyped for *ACE, IL-6, IL-8, VEGF* and *AGT* gene polymorphisms. Among these genotyped participants, 30 (13%) developed CIN. Controls (n=198) were participants not meeting CIN definition criteria as per KDIGO. The number of samples successfully genotyped for each of the SNPs was:

- For *ACE* (rs4646994), we had a total of 158 (69.3%) participants [30 (100%) CIN cases and 128 (64.6%) of controls].
- For *IL-6* (rs1800796), we had a total of 213 (93.4%) participants [30 (100%) CIN cases and 183 (92.4%) of controls].
- For *IL-8* (rs4073), we had a total of 205 (89.9%) participants [30 (100%) CIN cases and 175 (88.4%) of controls].
- For *VEGFA* (rs3025039), we had a total of 205 (89.9%) participants [30 (100%) CIN cases and 175 (88.4%) of controls].
- For *VEGF* (rs2010963), we had a total of 214 (93.9%) participants [30 (100%) CIN cases and 184 (92.9%) of controls].
- For *AGT* (rs699), we had a total of 214 (93.9%) participants [30 (100%) CIN cases and 184 (92.9%) of controls].

Table 3.1 illustrates the patients baseline characteristics. Ages ranged from 18-89 years with a median age of 53.5 years for CIN+ and 44 years for CIN- patients. Baseline serum creatinine and GFR were not significantly different between the CIN+ and CIN- groups. Compared to the control group (CIN-), CIN+ patients had significantly lower levels of haemoglobin and higher mortality rates. Post creatinine levels were higher in CIN+ patients than in CIN- patients (median (IQR) 98.5 µmol/L (70 – 132) vs 62.5 µmol/L (47 – 78.5); $p<0.001$).

Table 3.1 Participants' demographic and clinical characteristics

Characteristic	**CIN+ (30)**	**CIN− (198)**	***p* value** [a]
Age (years)	53.5 (39 – 62)	44 (33 – 57)	0.060
Gender (males: females)	16:14 (53.3% male)	82:74 (52.6% male)	0.938
HIV positive, *n* (%)	13 (48.2%)	39 (39.4%)	0.413
Mortality, *n* (%)	7 (24.1%)	11 (7.5%)	**0.007**
Hypertension, *n* (%)	8 (27.6%)	29 (19.5%)	0.324
Diabetes mellitus, *n* (%)	4 (13.8%)	14 (9.5%)	0.480
Baseline urea (mmol/L)	4.6 (4.1 – 6.5)	4.6 (3.5 – 5.8)	0.350
Baseline serum creatinine (μmol/L)	61 (51 – 100)	66 (51.5 – 84)	0.882
Baseline eGFR (ml/min/1.73 m^2) [b]	111.7 (89.5 – 139.7)	122.6 (96.4 – 139.9)	0.356
Baseline eGFR <60 ml/min/1.73 m^2, *n* (%)	2 (6.7%)	3 (1.5%)	0.073
Serum albumin (g/dL)	33 (27 – 37)	37 (30 – 40)	0.094
Haemoglobin (g/dL)	9.8 (8.3 – 11.9)	11.7 (9.2 – 13.8)	**0.006**
Systolic blood pressure (mmHg)	117 (106 – 134)	119 (110 – 128)	0.614
Diastolic blood pressure (mmHg)	76 (68 – 91)	71.5 (65 – 81)	0.228
Post creatinine [median (IQR)]	98.5 µmol/L (70 – 132)	62.5 µmol/L (47 – 78.5)	**<0.001**
Medications:			
ACEIs / ARBs	4 (13.8%)	16 (10.8%)	0.643
Statins	4 (13.8%)	12 (8.1%)	0.323
NSAIDs	1 (3.5%)	3 (2.0%)	0.638
NAC	0	2 (1.3%)	0.525
Diuretics	7 (24.1%)	24 (16.3%)	0.313

Abbreviations: HIV, human immunodeficiency virus; eGFR, estimated glomerular filtration rate; CIN, contrast-induced nephropathy; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; NSAID, nonsteroidal anti-inflammatory drug; NAC, N-acetyl cysteine; IQR, interquartile range.

[#] Median (IQR) given unless otherwise stated

[a] p-values calculated using a Wilcoxon–Mann–Whitney test for the continuous variables and a chi-square test for the categorical variables, comparing CIN positive and CIN negative participants.

[b] eGFR calculated using the CKD-epi formula.

Missing values as follows:

for CIN+ group: HIV n=3; mortality n=1; hypertension n=1; diabetes mellitus n=1; haemoglobin n=3; blood pressure n=11; ACEIs/ARBs n=1; statins n=1; NSAIDs n=1; diuretics n=1.

for CIN- group: age n=44; gender n=42; HIV n=99; mortality n=52; hypertension n=49; diabetes mellitus n=50; baseline urea n=47; baseline serum creatinine n=46; baseline eGFR n=46; serum albumin n=96; haemoglobin n=76; ACEIs/ARBs n=50; statins n=49; NSAIDs n=50; NAC n=48; diuretics n=51.

3.2. *ACE* (rs4646994) Results

3.2.1. Primer optimization

To optimize, 3 CIN- DNA samples were used to confirm the appropriate reaction conditions and binding of primers. The concentration of the DNA samples used was 10 ng/µl. The reaction setup with volumes and concentrations are illustrated in Tables 2.1 and 2.2 for PCR1 and PCR2, respectively.

3.2.2. *ACE* primer optimization results

The primers that were used in PCR1 and PCR2 reactions were previously described (Nakazawa *et al.*, 2012; Widodo *et al.*, 2017). In PCR1, each sample was classified into one of three possible genotypes: II (599 bp band), ID (599 bp and 312 bp bands) or DD (312 bp band). The DD genotypes were analysed again in PCR2 using insertion specific primers to observe if the I-allele amplicon (represented by 335 bp band) was present to avoid ID-DD mistyping. True DD samples did not amplify in PCR2.

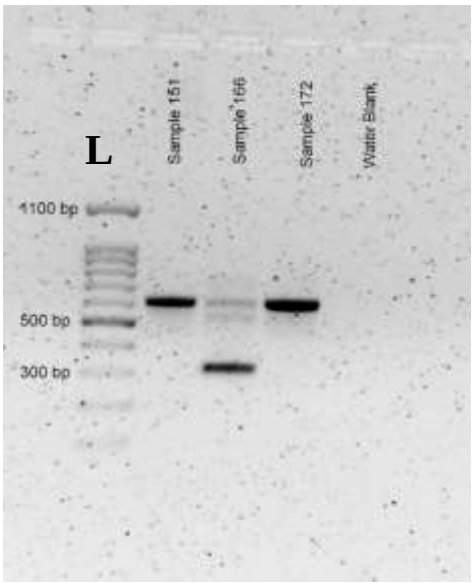


Figure 3.1a)

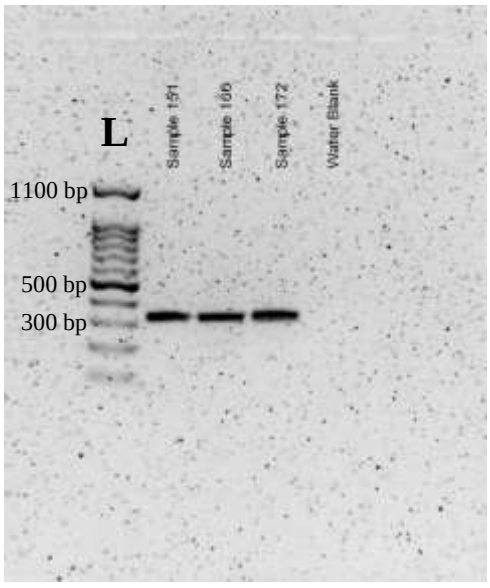


Figure 3.1b)

Figure 3.1a and 3.1b: A polymorphism analysis of the *ACE* SNP using PCR1 (Fig 3.1a) and PCR2 (Fig 3.1b). The genotypes were separated on a 1% agarose gel containing ethidium bromide. L-DNA ladder (100 bp); water blanks- negative controls (are both clear). Fig 3.1a: Sample 151 and 172- 599 bp band (II homozygote genotype); Sample 166- 312 bp and 599 bp bands (ID Heterozygote genotype). Fig 3.1b: Sample 151, 166 and 172- 335 bp band (I- allele). Therefore sample 151 and 172 represents the II homozygote genotype; sample 166 represents the ID heterozygote genotype.

All the samples amplified (Fig 3.1a and Fig3.1b), showing that the primer concentration of 0.5 µM was appropriate and the PCR reaction conditions were suitable with a final DNA volume of 25 ng and DNA polymerase of 0.5 U. No further optimization was required.

3.2.3. Conventional PCR results for the *ACE* SNP

Total DNA, extracted from 228 samples, was amplified as detailed in section 2.3.2.

Genotypes were recorded based on the electrophoresis patterns observed and each sample was classified into one of three possible genotypes: DD, ID, II as shown in Figure 3.2a and 3.2b. When no results were observed (sample failed to amplify) on the gel, the PCR was repeated for that sample and if it still failed to amplify, the sample was excluded from the analysis of this SNP. Figure 3.2a and 3.2b shows a representation of the polymorphism analysis of the *ACE* gene.

3.2.4. Association of the *ACE* SNP polymorphisms with CIN

To test whether the proportions were different in each group, we used a $\chi 2$ test of independence with $\alpha = 0.05$ as criterion for significance. The genotype and allele frequencies of the *ACE* SNP under the various inheritance models are illustrated in Table 3.2 (a-d). The genotype and allele frequencies of both the CIN and non-CIN groups are very similar and thus the distribution of observed values is very similar to that of expected values (Table 3.2d).

- **Genotypic association**

According to the χ^2 test of independence, this difference is not statistically significant, $p = 0.890$, for the basic distribution model (Table 3.2a); $p = 0.939$ for the dominant model (Table 3.2b); $p = 0.651$ for the recessive model (Table 3.2c). The *p*-values presented in Table 3.2a-c are all above 0.05 and indicate no association between rs4646994 and CIN at the genotypic level.

- **Allelic association**

The *p*-value obtained for the allelic association ($p = 0.747$) presented in Table 3.2d is above 0.05 and indicate that there is no significant association. A regression model was performed to assess the additive association of CIN with the *ACE* SNP. The test results illustrate no association, $p = 0.780$ (Table 3.2e).

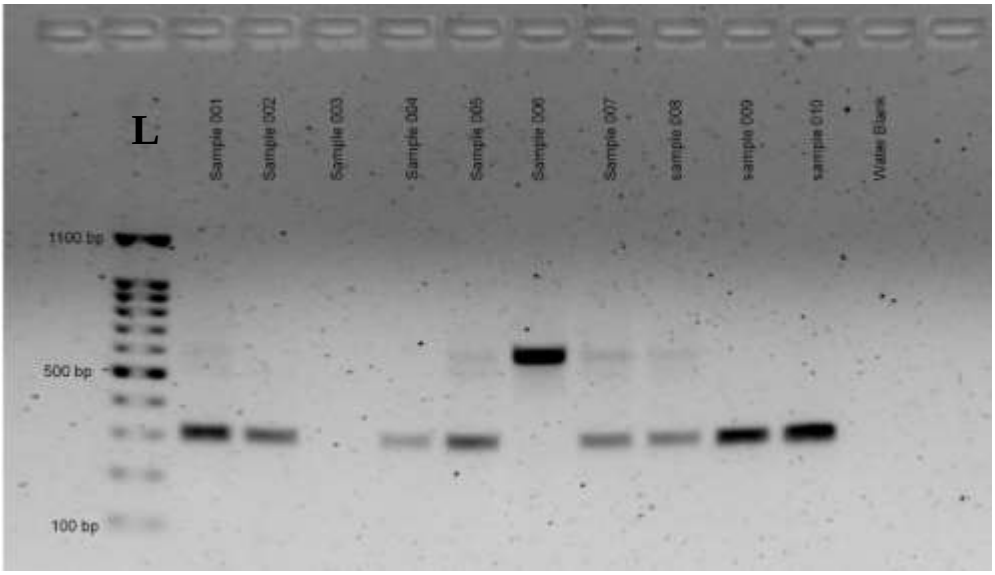


Figure 3.2a)

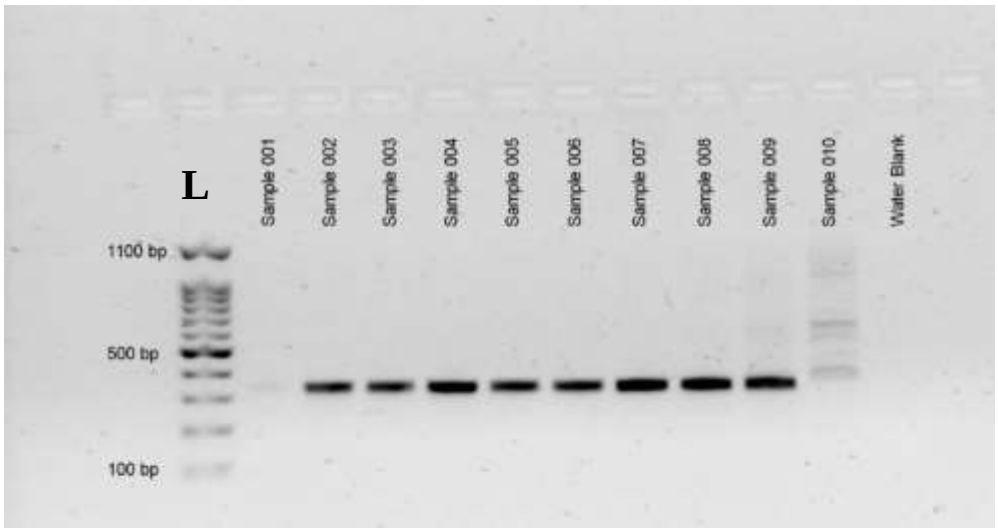


Figure 3.2b)

Figure 3.2a and 3.2b: A Polymorphism analysis of the *ACE* genotypes using PCR1 (Fig 3.2a) and PCR2 (Fig 3.2b). The genotypes were separated on a 1% agarose gel containing ethidium bromide. L-DNA ladder (100 bp); water blank- negative controls. Genotypes for Fig 3.2a are as follows: Sample 001, 002, 004, 009, 010-312 bp band (DD homozygote); sample 005, 007, 008- 312 bp and 599 bp bands (ID heterozygote); sample 006-599 bp band (II homozygote); Sample 003- failed to amplify. Fig 3.2b genotypes: Sample 001 and 010-DD homozygote (sample 010 displays non-specific binding); sample 002, 003, 004, 005, 007, 008, 009- 335 bp band (I allele) thus sample 002, 004, 005, 007-009 (ID heterozygote), sample 006 (II homozygote); sample 003 needs repeat of PCR 1. The CIN positive group was consistent with HWE, $p = 0.17$. However, there was extensive deviation from HWE ($p < 0.05$) for the non-CIN group ($p = 0.00015$) and overall genotype sample size ($p = 0.000059$).

Table 3.2 Association of *ACE* (rs4646994) polymorphism with CIN

	CIN cases (*N*=30)	**non-CIN (*N*=128)**	***p*-value**	**χ^2 value (df)**
(a) Basic distribution				
II	5 (16.7%)	26 (20.3%)	0.890	0.232 (2)
ID	10 (33.3%)	39 (30.5%)		
DD	15 (50%)	63 (49.2%)		
(b) Dominant Model				
ID or II	15 (50%)	65 (50.8%)	0.939	0.0059 (1)
DD	15 (50%)	63 (49.2%)		
(c) Recessive Model				
DD or ID	25 (83.3%)	102 (79.7%)	0.651	0.2048 (1)
II	5 (16.7%)	26 (20.3%)		

CIN: Contrast-Induced Nephropathy, χ^2 value (df): Chi-square statistic (degrees of freedom),

***p*<0.05**

(d) Multiplicative Model- Analysed by alleles

Observed

	CIN	**non-CIN**	
I	20	91	111
D	40	165	205
	60	256	316

Expected

	CIN	**non-CIN**	
I	21.076	89.924	111
D	38.924	166.08	205
	60	256	316

Chi2 *p*-value= 0.747

(e) Additive Model

SNP	***β*- Coef**	**95% CI**	***p*= value**
rs4646994	0.011	-0.0686398; 0.091	0.780

SNP: Single nucleotide polymorphism, *β*- Coef: Beta Coefficient-, CI: Confidence Interval, ***p*<0.05**

3.3. Real-time PCR (qPCR) results

3.3.1. Genotyping of *IL-6, IL-8, VEGFA, VEGF* and *AGT* SNPs

The process of determining the genotypes of the CIN and non-CIN (control) cases (N=228) for the *IL-6, IL-8, VEGFA, VEGF* and *AGT* SNPs involved two steps: DNA extraction followed by Real-Time PCR where the amplification of all the polymorphisms was monitored, and detection of fluorescence signal was performed using the PikoReal-Real-Time PCR System software version 2.2. Confirmatory sequencing was done on a few samples from each SNP to validate the genotypes. Electropherograms are depicted in colour with each nucleotide base represented by a different colour.

3.3.2. Primer/Probe optimization

To optimize the primer/probe mix, a total of 15 DNA samples from the study were used to establish the appropriate reaction conditions and to ascertain whether the primer/probe mix was effective for SNPs rs1800796, rs4073, rs3025039, rs2010963, and rs699. In total, 25 ng of each DNA sample was used. The PCR reaction setup with volumes and concentrations are shown in Table 2.5.

- **Primer/Probe optimization results analysis**

The results of the primer/probe mix optimization for all the SNPs were identified using allelic discrimination (AD) assay graphs. The AD assay graphs show groups or clusters of samples indicating the three different genotypes for each SNP:

- For *IL-6* (rs1800796)- GG, GC, or CC
- For *IL-8* (rs4073)- AA, AT, or TT
- For *VEGF* (rs3025039)-CC, CT, or TT
- For *VEGF* (rs2010963)- GG, GC, or CC
- For *AGT* (rs699)- GG, GA, or AA

Samples that failed to amplify were repeated, and if they once again failed to amplify, they were excluded from the analysis.

3.3.3. Sequencing primer optimization results of SNPs

A total of 3 DNA samples were used to optimize the PCR reactions to be sent for sequencing for SNPs rs1800796, rs4073, rs3025039, rs2010963, and rs699.The DNA sample concentration used

was 10 ng/µl. The PCR reaction conditions with volumes and concentrations were the same as those illustrated in Table 2.1, with the only difference being the use of specific forward and reverse primers (IDT) illustrated in (Table 2.6) for each SNP.

- **Primer optimization results**

The results of the primer mix optimization for all the SNPs illustrated that the samples amplified at a primer concentration of 500 nM and no further optimization was required (Figure 3.3).

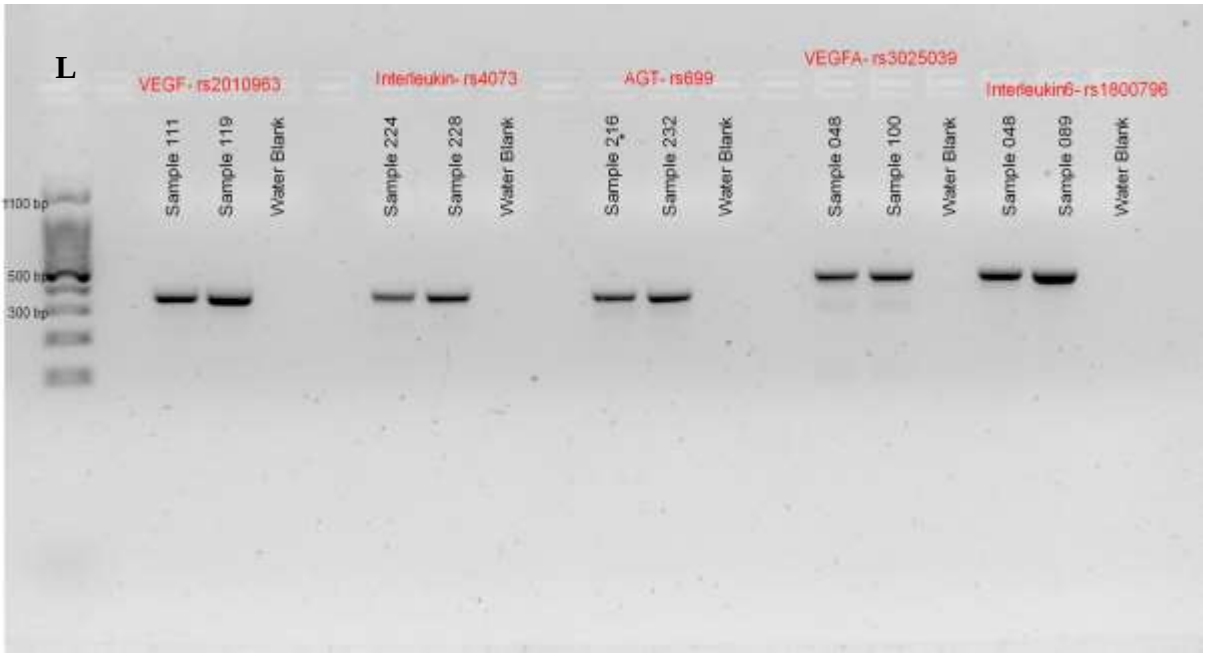


Figure 3.3: Gel photograph illustrating DNA products of various control samples that amplified for SNPs rs2010963, rs4073, rs699, rs3025039, and rs1800796. The PCR products were run on a 1% agarose gel containing ethidium bromide. L-DNA ladder (100 bp); water blanks- negative controls (no DNA).

3.3.4. Statistical analysis

3.3.4.1. Association of *IL-8, IL-6, VEGFA, VEGF* and *AGT* SNPs with CIN

Genotypes at each locus were scored in all individuals and their frequencies were computed using direct gene counting. The genotypic and allelic distributions of the determined polymorphisms are illustrated in Tables 3.3-3.7.

3.3.4.2. Association testing

Tables 3.3-3.7 below indicate the results generated from χ^2 test of independence with $\alpha = 0.05$ as level of significance or with a Fisher's exact test when frequencies in any group were less than 5, which both looked for association between the polymorphic variants and the susceptibility to CIN.

3.3.5. SNP results

3.3.5.1. *IL-6* (rs1800796)

The primer/probe optimization results showed that the samples amplified (Figure 3.4) adequately and thus no further optimization was required. The following AD graph (Figure 3.5) represents the findings for SNP rs1800796 showing the three distinct genotype populations. Sequencing was performed to confirm the results observed through the AD graphs. These sequencing results are depicted as a series of electropherograms in Figure 3.6.

- **Statistical analysis**

Using the χ^2 test of independence, we found that there are no significance differences in genotype and allele frequencies between CIN cases and controls for the rs1800796 SNP. The distribution of genotype and allele frequencies are illustrated in Table 3.3. The genotype and allele frequencies indicate a no statistically significant difference, $p = 0.591$ for the dominant model (Table 3.3b). The *p*-values obtained for the genotype (Fisher's exact test) and recessive model tests performed on rs1800796 SNP are greater than 0.05 (Tables 3.3a and 3.3c). Table 3.3d illustrates a lack of allelic association with CIN ($p = 0.764$). A regression model was performed to further assess the additive association between CIN and the *IL-6* SNP, $p = 0.769$ (Table 3.3e), which was not significant. The *IL-6* SNP was consistent with HWE ($p > 0.05$), the CIN positive group ($p = 0.47$), non-CIN group ($p = 0.41$) and overall genotype sample size ($p = 0.65$).

Primer/Probe Optimization Results

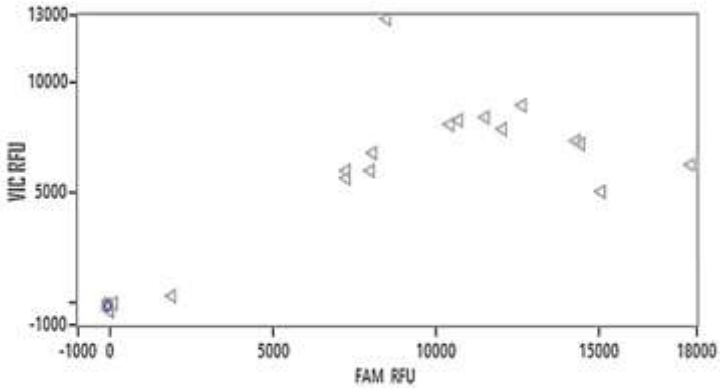


Figure 3.4: TaqMan allelic discrimination assay of *IL-6* SNP demonstrating amplification of samples with the primer/probe mix.

AD Graph Analysis

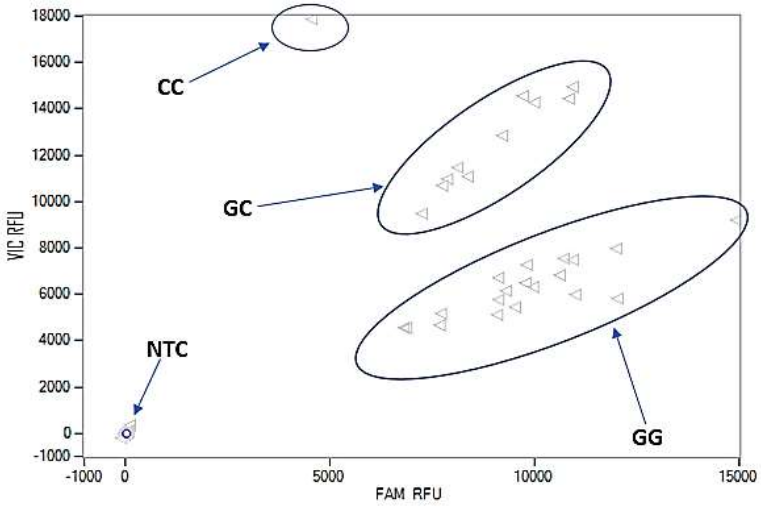


Figure 3.5: Two parameter amplification plots demonstrating the separation of the three genotype populations and NTC- No template controls (lower left population) for *IL-6* (rs1800796) polymorphism. FAM and VIC are represented by allele G and C, respectively.

Sequencing Results

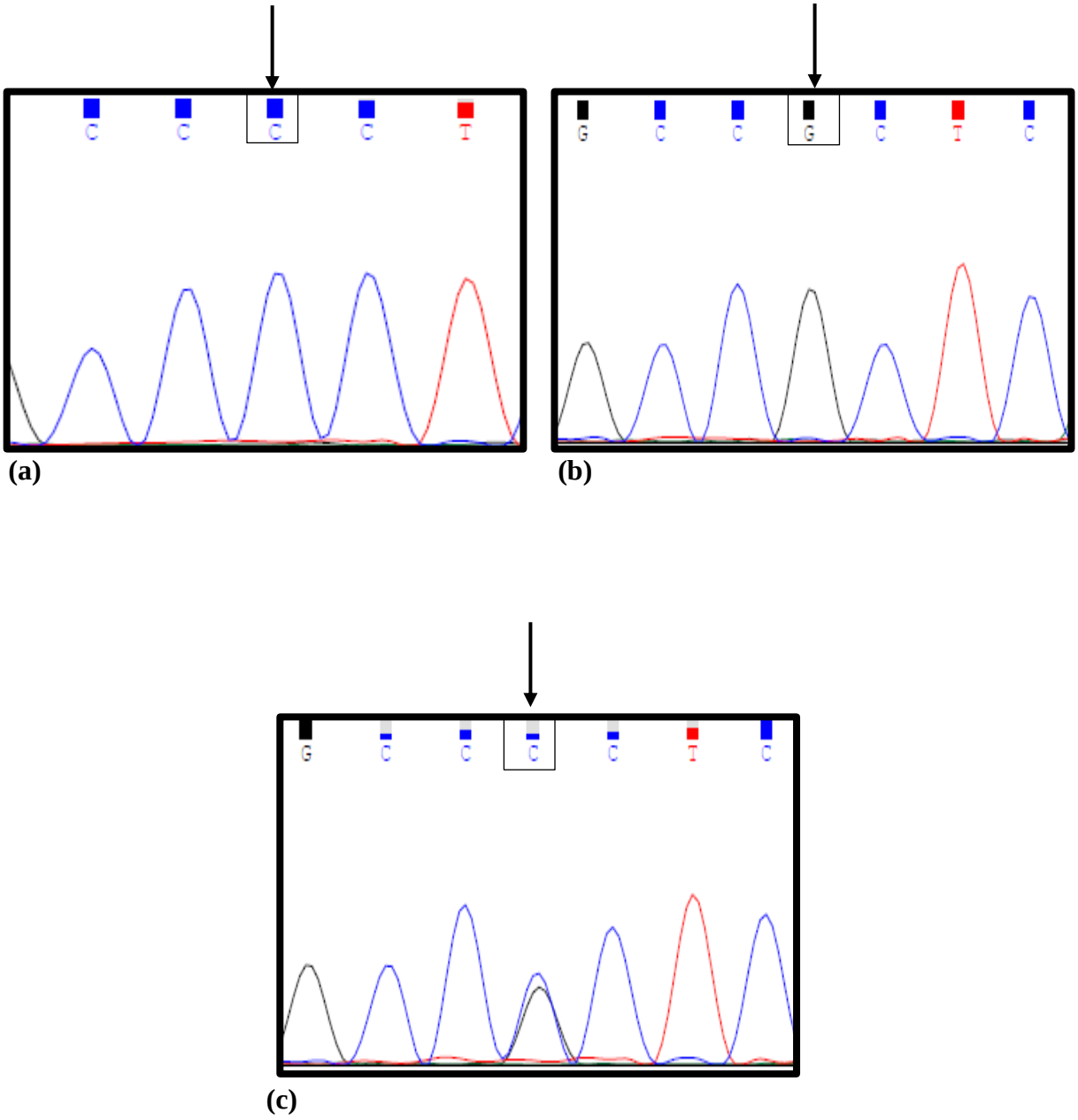


Figure 3.6: Sequencing results confirming the rs1800796 (*IL-6*) genotypes. The arrows indicate the position of the SNP. These forward strands represents CC homozygote: Control sample 265 (a), GG homozygote: Control sample 089 (b), and GC heterozygote: Control sample 070 (c). At position "C", the strand illustrates a GC heterozygote (blue and black double peak).

Table 3.3 Association of *IL-6* (rs1800796) polymorphism with CIN by genetic models

	CIN cases (*N*=30)	**non-CIN (*N*=183)**	***p*-value**	**χ^2 value (df)**	**Fisher's exact *p*-value**
(a) Basic distribution					
GG	23 (76.7%)	148 (80.9%)			0.655
GC	7 (23.3%)	32 (17.5%)			
CC	0 (0%)	3 (1.6%)			
(b) Dominant Model					
GC or CC	7 (23.3%)	35 (19.1%)	0.591	0.2883 (1)	
GG	23 (76.7%)	148 (80.9%)			
(c) Recessive Model					
GG or GC	30 (100%)	180 (98.4%)			1.000
CC	0 (0%)	3 (1.6%)			

CIN: Contrast-Induced Nephropathy, χ^2 value (df): Chi-square statistic (degrees of freedom),

***p*<0.05**

(d) Multiplicative Model- Analysed by alleles

Observed

	CIN	**non-CIN**	
C	7	38	45
G	53	328	381
	60	366	426

Expected

	CIN	**non-CIN**	
C	6.338	38.662	45
G	53.662	327.34	381
	60	366	426

Chi2 *p*-value= 0.764

(e) Additive Model

SNP	***β*- Coef**	**95% CI**	***p*= value**
rs1800796	0.016	-0.0909825; 0.123	0.769

SNP: Single nucleotide polymorphism, *β*- Coef: Beta Coefficient-, CI: Confidence Interval, ***p*<0.05**

3.3.5.2. *IL-8* (rs4073)

The primer/probe optimization results illustrated in Figure 3.7 indicate that the samples amplified well. Figure 3.8 illustrates the various genotypes indicated by the three populations/clusters produced by the AD graphs. Electropherograms confirming genotype results observed through AD graphs are represented in Figure 3.9.

- **Statistical analysis**

The χ^2 test of independence and Fisher's exact association tests were performed to confirm the above observations. Table 3.4a-d illustrates *p*- values obtained for the genotype and allele tests performed on rs4073. The *IL-8* SNP results show no association with CIN at either the allelic (Table 3.4d, $p = 0.249$) or genotypic level, $p = 0.103$ for the dominant model (Table 3.4b). Using the Fisher's exact, we found no statistical significance ($p > 0.05$) for the basic distribution (Table 3.4a, $p = 0.133$) or the recessive model [Table 3.4c, $p = 1.000$]. For further association testing with the additive model, a regression analysis was performed, and the results indicate no association, $p = 0.238$. The *IL-8* SNP was consistent with HWE ($p > 0.05$), the CIN positive group ($p = 0.10$), non-CIN group ($p = 0.94$) and overall genotype sample size ($p = 0.43$).

Primer/Probe Optimization Results

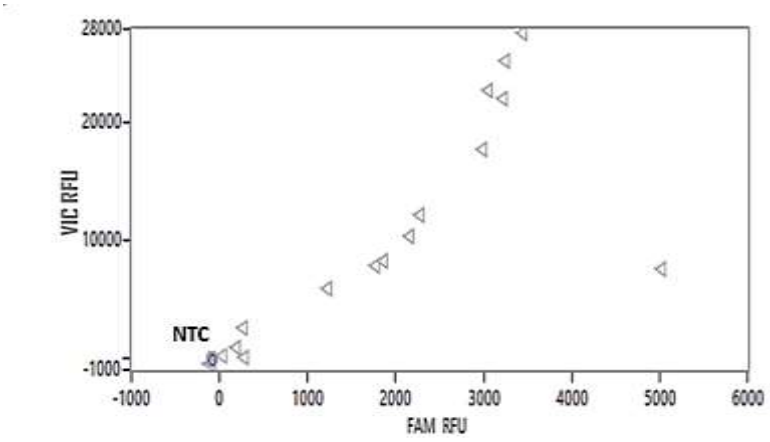


Figure 3.7: Allelic discrimination graph illustrating the samples that amplified when testing primer/probe concentration mix for *IL-8* SNP.

AD Graph Analysis

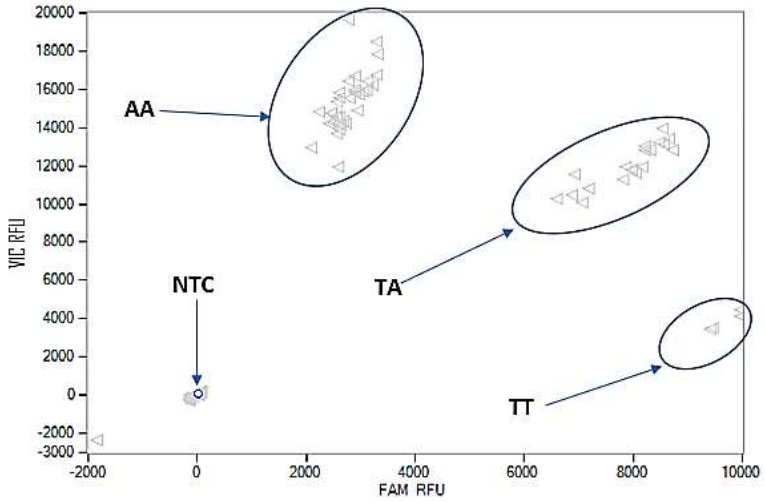
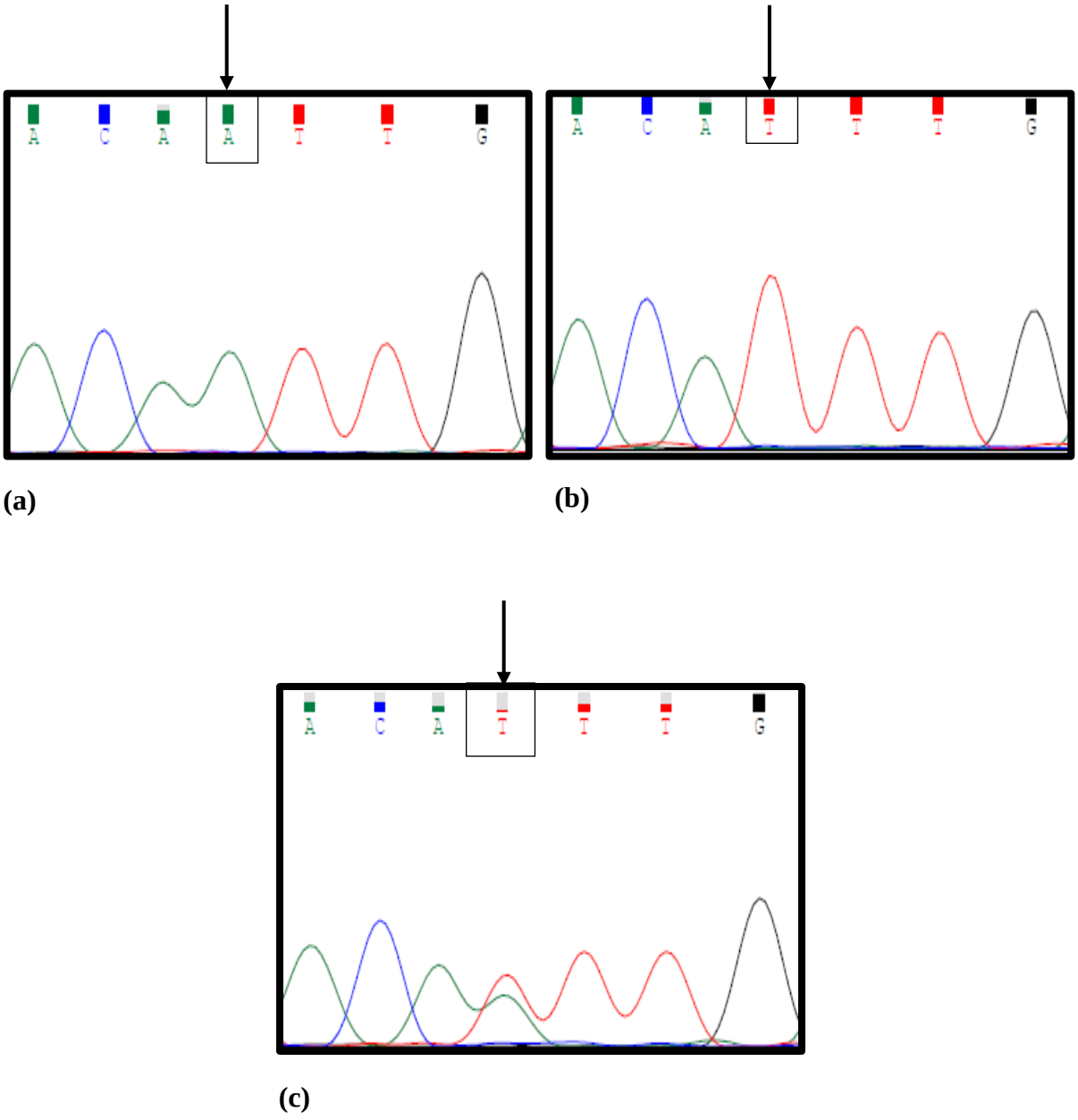


Figure 3.8: Allelic discrimination assay illustrating the separation of the three genotype populations and NTC- No template controls for *IL-8* (rs4073) polymorphism. T and A alleles represent florophores FAM and VIC, respectively.

Sequencing Results



(a) (b)

(c)

Figure 3.9: Electropherograms showing sequence data from individuals (a) Homozygous for the "A" allele: AA genotype, (b) homozygous for the "T" allele: TT genotype, and TC heterozygous (c), from the sequencing in the forward directions (arrows indicate SNP position). A green and red double peak, indicating both "A" and "T" alleles can be seen on the forward strand (c).

Table 3.4 Association of *IL-8* (rs4073) polymorphism with CIN by genetic models

	CIN cases (*N*=30)	**non-CIN (*N*=175)**	***p*-value**	**χ^2 value (df)**	**Fisher's exact *p*-value**
(a) Basic distribution					
AA	16 (53.3%)	120 (68.6%)			0.133
AT	14 (46.7%)	50 (28.6%)			
TT	0 (0%)	5 (2.9%)			
(b) Dominant Model					
AT or TT	14 (46.7%)	55 (31.4%)	0.103	2.6631 (1)	
AA	16 (53.3%)	120 (68.6%)			
(c) Recessive Model					
AA or AT	30 (100%)	170 (97.1%)			1.000
TT	(0%)	5 (2.9%)			

CIN: Contrast-Induced Nephropathy, χ^2 value (df): Chi-square statistic (degrees of freedom)

***p<0.05**

(d) Multiplicative Model- Analysed by alleles

Observed

	CIN	**non-CIN**	
T	14	60	74
A	46	290	336
	60	350	410

Expected

	CIN	**non-CIN**	
T	10.829	63.171	74
A	49.171	286.83	336
	60	350	410

Chi2 *p*-value= 0.249

(e) Additive Model

SNP	***β*- Coef**	**95% CI**	***p*= value**
rs4073	0.055	-0.036862; 0.148	0.238

SNP: Single nucleotide polymorphism, *β*- Coef: Beta Coefficient-, CI: Confidence Interval, ***p*<0.05**

3.3.5.3. *VEGFA* (rs3025039)

Figure 3.10 shows the primer/probe optimization results demonstrating amplification of samples with the TaqMan primer/probe mix. No further optimization was required. Figure 3.11 depicts the AD graph demonstrating various genotype populations. Sequencing results are represented by a series of electropherograms confirming the genotype results produced by AD graphs (Figure 3.12).

- **Statistical analysis**

The Fisher's Exact test was performed to compare genotype or allele counts between CIN and non- CIN cases. Allele and genotype tests using different genetic modes of association were performed to look for association between *VEGFA* SNP and CIN. Table 3.5 illustrates the *p*- values calculated for the allelic and genotypic associations. The *p*- values are all above 0.05 (Table 3.5a-d) and indicate no significant association. A regression model was performed for further association testing under the additive model. The results indicate no association, $p = 0.238$ (Table 3.5e). The *VEGFA* SNP was consistent with HWE ($p > 0.05$), the CIN positive group ($p = 0.85$), non-CIN group ($p = 0.96$) and overall genotype sample size ($p = 0.98$).

Primer/Probe Optimization Results

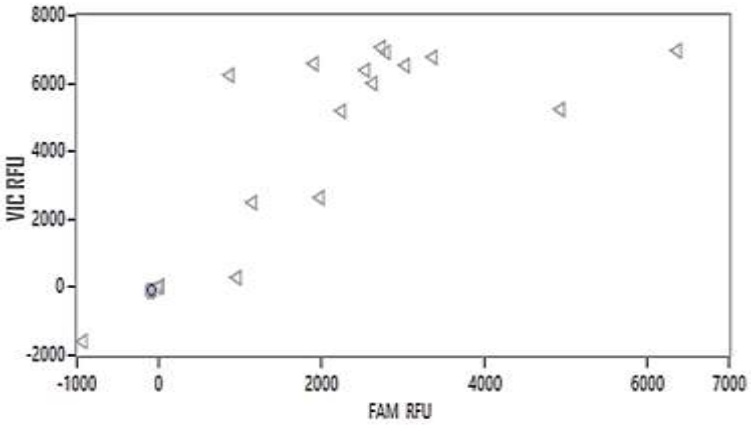


Figure 3.10: Primer/probe optimization results of *VEGFA* SNP (rs3025039) produced by TaqMan AD graph illustrating sample amplification.

AD Graph Analysis

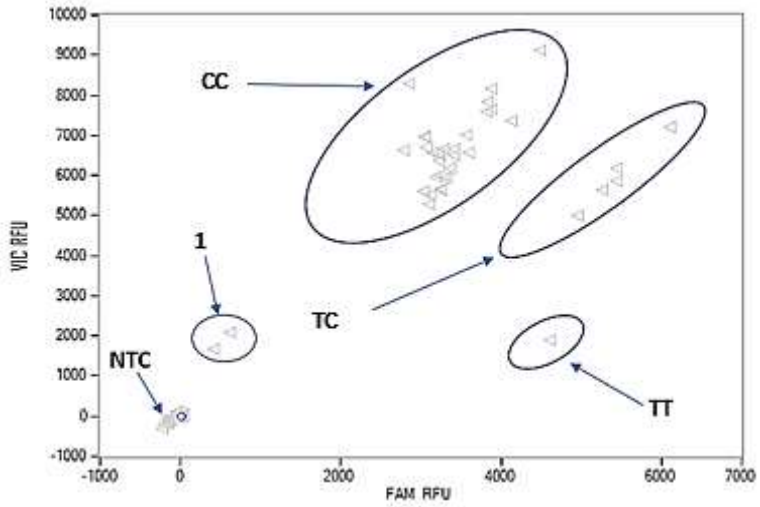


Figure 3.11: Two parameter amplification plots showing the separation of the genotype populations and NTC (negative controls) for *VEGFA* SNP (rs3025039). Samples labelled 1- failed to amplify. FAM- "T" allele and VIC- "C" allele.

Sequencing Results

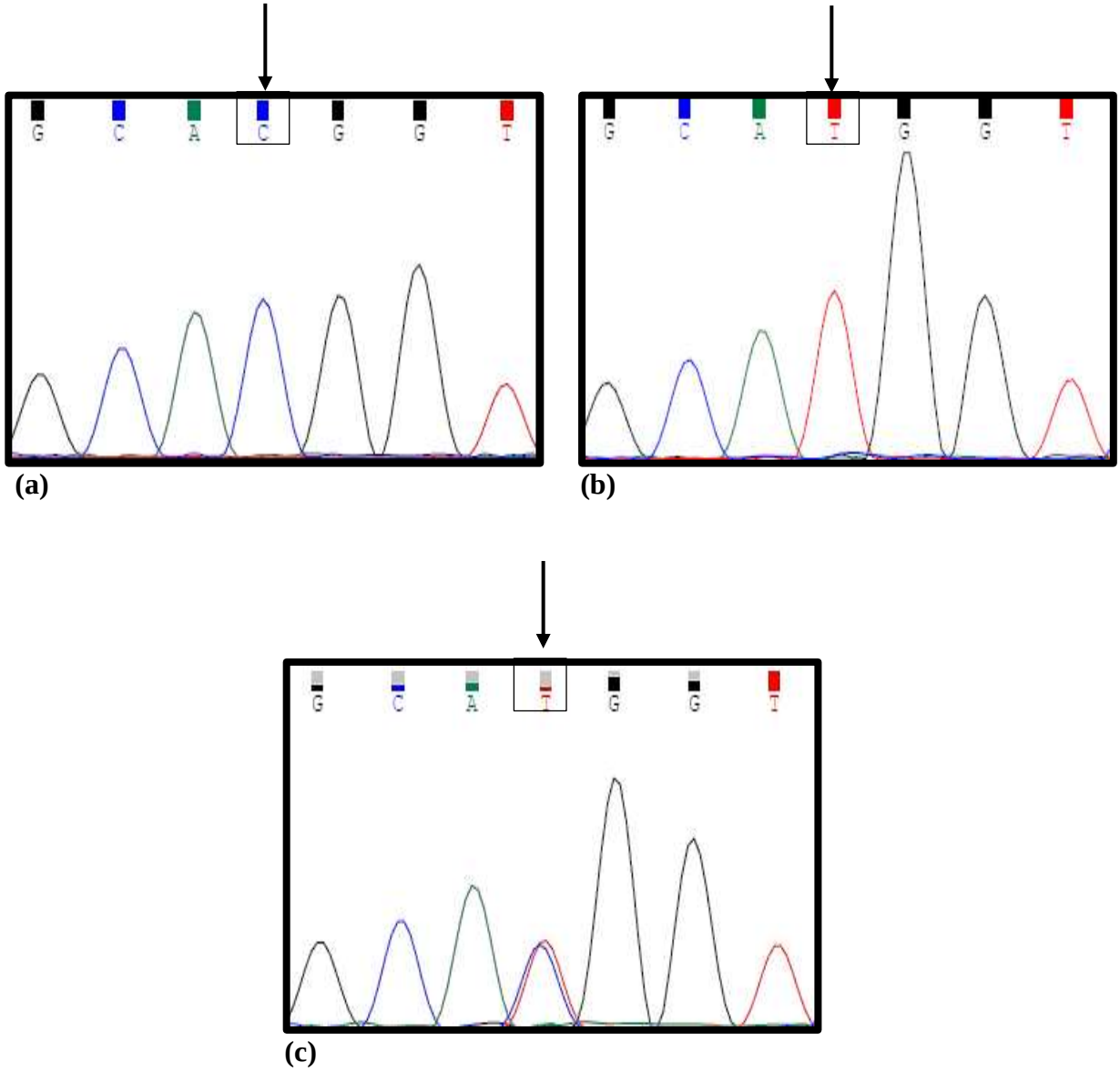


Figure 3.12: Electropherograms (forward strands) confirming genotyping results obtained from AD graphs of rs3025039 (*VEGFA*). The arrows indicate the position of the SNP. CC homozygote: Control sample 175 (a). TT homozygote: Control sample 022 (b). TC heterozygote: DNA sample sequenced was control sample 277 (c). At position "T", the strand indicates a TC heterozygote (blue and red double peak).

Table 3.5 Association of *VEGFA* (rs3025039) polymorphism with CIN using genetic modes of association

	CIN cases (*N*=30)	**non-CIN (*N*=175)**	**Fisher's exact *p*-value**
(a) Basic distribution			
CC	28 (93.3%)	149 (85.1%)	0.473
CT	2 (6.7%)	25 (14.3%)	
TT	0 (0%)	1 (0.6%)	
(b) Dominant Model			
CT or TT	2 (6.7%)	26 (14.9%)	0.386
CC	28 (93.3%)	149 (85.1%)	
(c) Recessive Model			
CC or CT	30 (100%)	174 (99.4%)	1.000
TT	0 (0%)	1 (0.6%)	

CIN: Contrast-Induced Nephropathy, ***p*<0.05**

(d) Multiplicative Model- Analysed by alleles

Observed

	CIN	**non-CIN**	
C	58	323	381
T	2	27	29
	60	350	410

Expected

	CIN	**non-CIN**	
C	55.7561	325.2439	381
T	4.243902	24.7561	29
	60	350	410

Chi2 *p*-value= 0.221

(e) Additive Model

SNP	***β*- Coef**	**95% CI**	***p*= value**
rs3025039	-0.083	-0.2179636; 0.051	0.223

SNP: Single nucleotide polymorphism, *β*- Coef: Beta Coefficient-, CI: Confidence Interval, ***p*<0.05**

3.3.5.4. *VEGF* (rs2010963)

The primer/probe optimization results demonstrating amplification of samples are illustrated in Figure 3.13. The primer/probe mix was optimal and thus no further optimization was required. Figure 3.14 represents genotype results produced by the AD graph demonstrating three various genotype populations. Figure 3.15 illustrates sequencing results produced for the GC heterozygote genotype.

- **Statistical analysis**

Using the χ^2 test of independence, we performed the case/control analysis considering the three models of association. The genotype and allele frequencies of *VEGF* SNP are presented in Tables 3.6a-d. The *p*-values observed are all above 0.05 indicating no association, $p = 0.518$ for the basic distribution model (Table 3.6a); $p = 0.740$ for the dominant model (Table 3.6b); $p = 0.359$ for the recessive model (Table 3.6c). To further confirm a lack of additive CIN association with rs2010963 SNP, a standard regression model was performed. The results illustrate no association at both the allelic and genotypic level, $p = 0.851$. The *VEGF* SNP was consistent with HWE ($p > 0.05$), the CIN positive group ($p = 0.17$), non-CIN group ($p = 0.71$) and overall genotype sample size ($p = 0.39$).

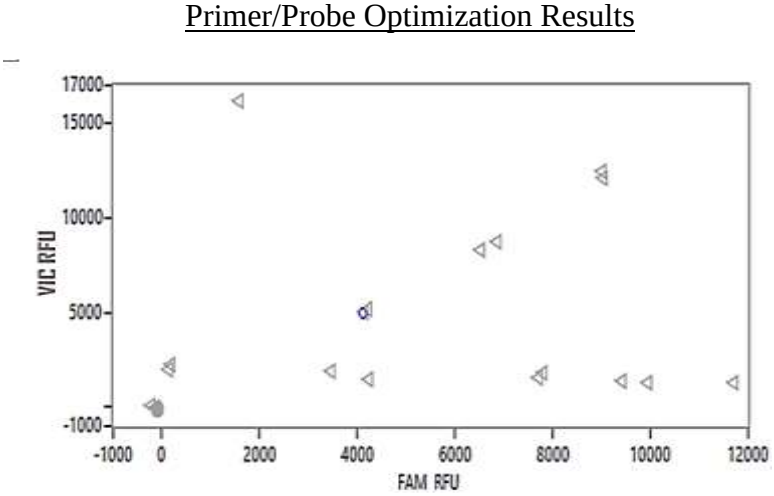


Figure 3.13: AD graph demonstrating amplification of samples at a primer/probe mix of 1x for the *VEGF* SNP.

AD Graph Analysis

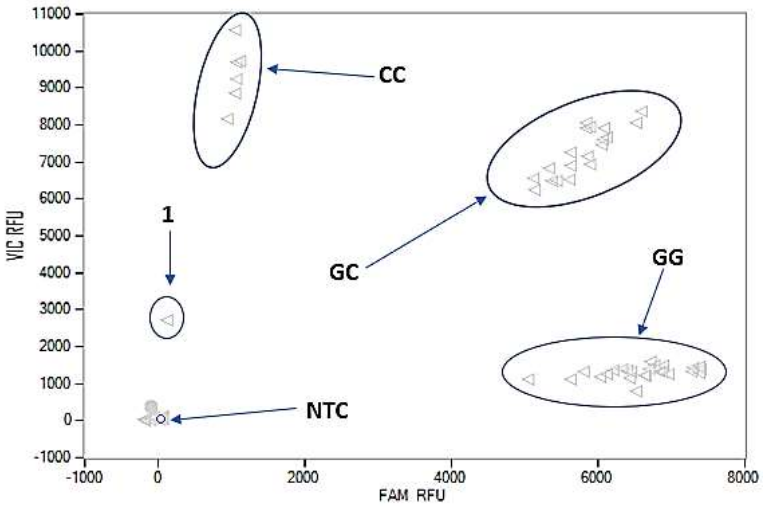


Figure 3.14: AD graphs demonstrating the separation of various genotype populations and NTC for *VEGF* (rs2010963) polymorphism. Sample labelled 1- failed to amplify. FAM (G-allele) and VIC (C-allele).

Sequencing Results

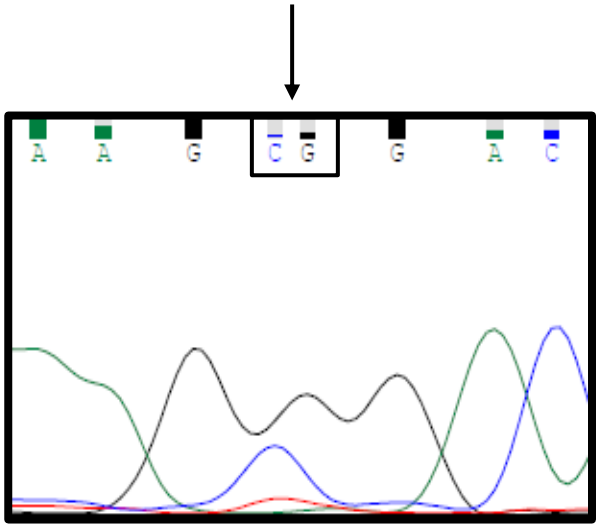


Figure 3.15: Chromatograph (forward strand only) confirming rs2010963 genotype. The arrows indicate the position of the SNP. This Figure indicates a GC heterozygote (black and blue double peak).

Table 3.6 Association of *VEGF* (rs2010963) polymorphism with CIN using genetic models

	CIN cases (*N*=30)	**non-CIN (*N*=184)**	***p*-value**	**χ2 value (df)**
(a) Basic distribution				
GG	15 (50%)	86 (46.7%)	0.518	1.3148 (2)
GC	10 (33.3%)	78 (42.4%)		
CC	5 (16.7%)	20 (10.9%)		
(b) Dominant Model				
GC or CC	15 (50%)	98 (53.3%)	0.740	0.1101 (1)
GG	15 (50%)	86 (46.7%)		
(c) Recessive Model				
GG or GC	25 (83.3%)	164 (89.1%)	0.359	0.8402 (1)
CC	5 (16.7%)	20 (10.9%)		

CIN: Contrast-Induced Nephropathy, χ^2 value (df): Chi-square statistic (degrees of freedom)

***p<0.05**

(d) Multiplicative Model- Analysed by alleles

Observed

	CIN	**non-CIN**	
C	20	118	138
G	40	250	290
	60	368	428

Expected

	CIN	**non-CIN**	
C	19.34579	118.6542	138
G	40.65421	249.3458	290
	60	368	428

Chi2 *p*-value= 0.845

(e) Additive Model

SNP	***β*- Coef**	**95% CI**	***p*= value**
rs2010963	0.006	-0.0624888; 0.076	0.851

SNP: Single nucleotide polymorphism, *β*- Coef: Beta Coefficient, CI: Confidence Interval, ***p<0.05**

3.3.5.5. *AGT* (rs699)

Figure 3.16 below illustrates the results of the primer/probe mix optimization demonstrating amplification of samples. Figure 3.17 is demonstrating genotype results produced by the AD graphs and figure 3.18 illustrates sequencing results produced for the various genotypes.

- **Statistical analysis**

Using the χ^2 test of independence, we found no significant genotypic association, $p = 0.989$ for the dominant model (Table 3.7b). In addition, a Fisher's Exact test was used to further asses the association between rs699 SNP and CIN. The Fisher's Exact *p*-values for the basic distribution (Table 3.7a) and recessive model (Table 3.7c) are both above 0.05 indicating no significant association. Table 3.7e indicate no association with the additive model, $p = 0.724$. The CIN positive group was consistent with HWE, $p = 0.54$. However, there was deviation from HWE ($p < 0.05$) for the non-CIN group ($p = 0.0127$) and overall genotype sample size ($p = 0.0312$).

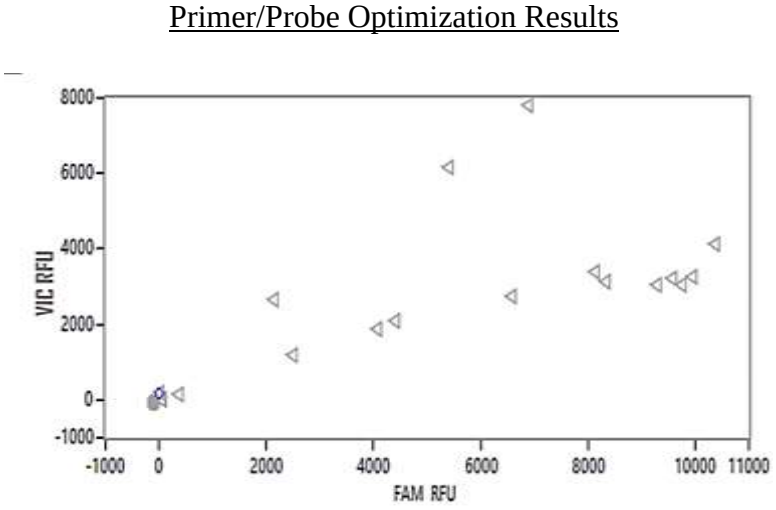


Figure 3.16: Allelic discrimination plot demonstrating sample amplification for the *AGT* SNP.

AD Graph Analysis

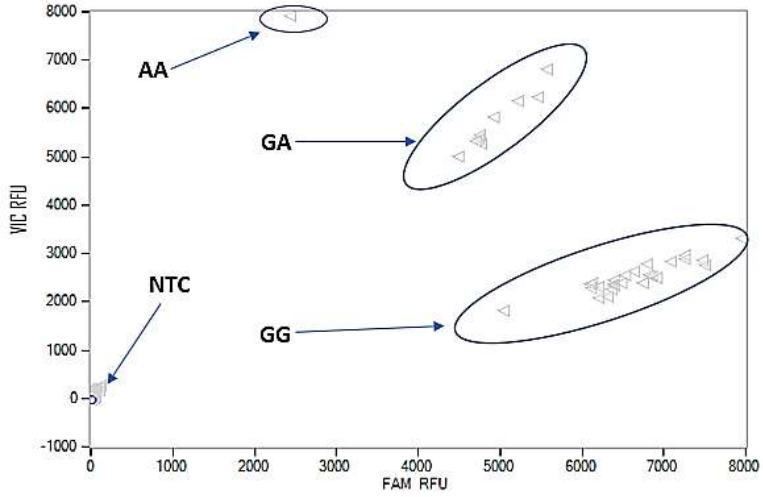
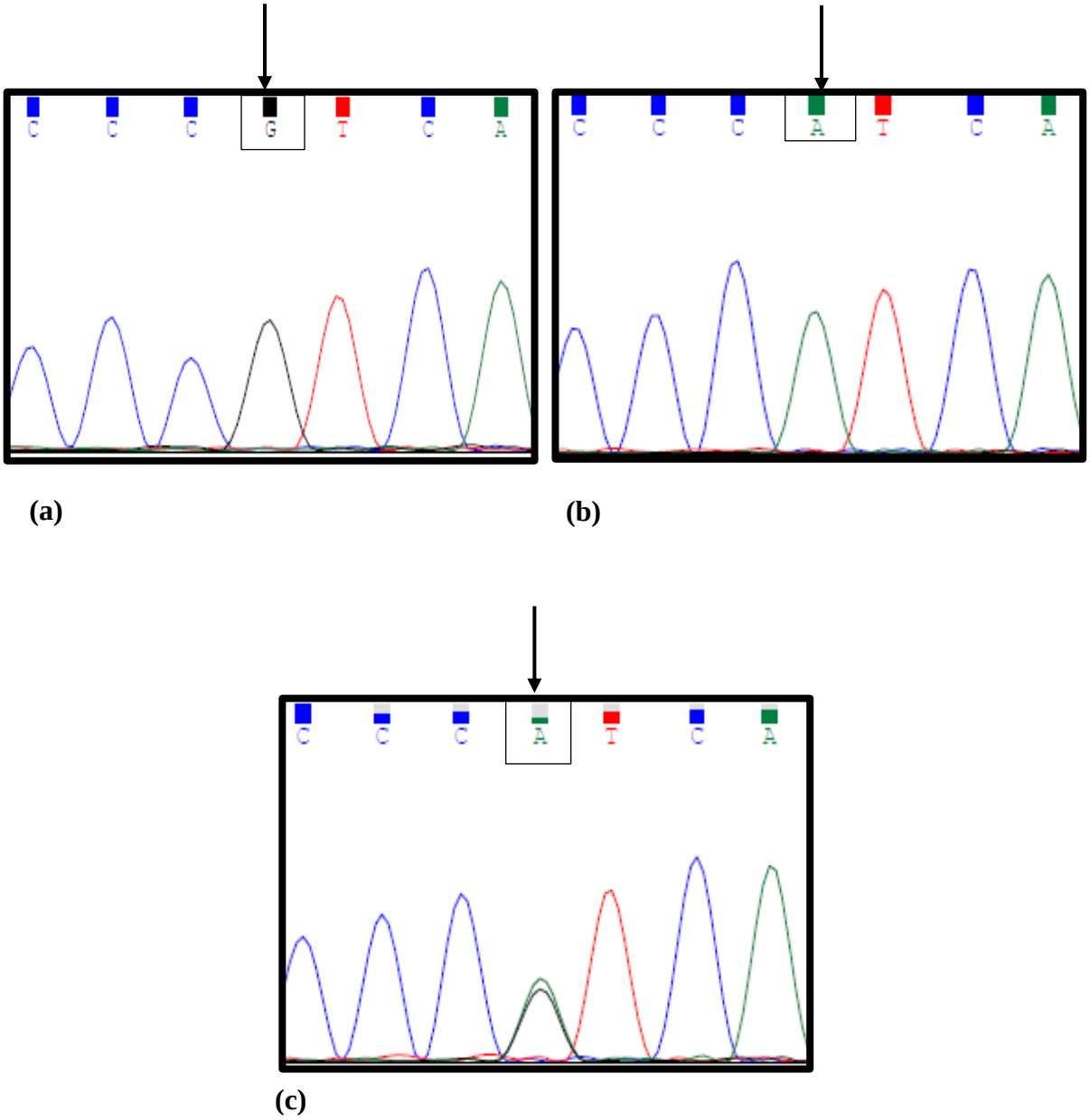


Figure 3.17: Two parameter amplification graph demonstrating the different genotype populations and NTC (No DNA) for *AGT* (rs699) polymorphism. FAM and VIC flourophores are represented by alleles G and A, respectively.

Sequencing Results



(a) **(b)**

(c)

Figure 3.18: Sequencing results of the forward strands confirming the three genotypes obtained for rs699. Figure 3.18a visualizes a single black peak indicative of a "G" allele (GG homozygous), (b) a single green peak represents AA homozygous genotype. A black and green double peak illustrating the presence of both the "G" and "A" alleles is present on the forward strand (C). The arrows indicate SNP position.

Table 3.7 Association of *AGT* (rs699) polymorphism with CIN using genetic models

	CIN cases (*N*=30)	**non-CIN (*N*=184)**	***p*-value**	**χ^2 value (df)**	**Fisher's exact *p*-value**
(a) Basic distribution					
GG	24 (80%)	147 (79.9%)			0.762
GA	6 (20%)	31 (16.8%)			
AA	0 (0%)	6 (3.3%)			
(b) Dominant Model					
GA or AA	6 (20%)	37 (20.1%)	0.989	0.0002 (1)	
GG	24 (80%)	147 (79.9%)			
(c) Recessive Model					
GG or GA	30 (100%)	178 (96.7%)			0.598
AA	0 (0%)	6 (3.3%)			

CIN: Contrast-Induced Nephropathy, χ^2 value (df): Chi-square statistic (degrees of freedom),

***p<0.05**

(d) Multiplicative Model- Analysed by alleles

Observed

	CIN	**non-CIN**	
G	54	325	379
A	6	43	49
	60	368	428

Expected

	CIN	**non-CIN**	
G	53.13084	325.8692	379
A	6.869159	42.13084	49
	60	368	428

Chi2 *p*-value= 0.704

(e) Additive Model

SNP	***β*- Coef**	**95% CI**	***p*= value**
rs699	-0.017	-0.1148853; 0.08	0.724

SNP: Single nucleotide polymorphism, *β*- Coef: Beta Coefficient-, CI: Confidence Interval, ***p*<0.05**

Chapter 4

4. DISCUSSION

In previous studies, investigators reported that inflammation plays a crucial role in the pathogenesis of renal disease including AKI (Kinsey *et al*., 2008; Akcay *et al.,* 2009; Ortega & Fornoni, 2010; Sharfuddin & Molitoris, 2011). Polymorphisms in the genes involved in inflammatory response may influence the development of AKI by altering the level of inflammation in the body. For this reason, we investigated the association of six polymorphisms located in five inflammation related genes with iatrogenic acute kidney injury also known as CIN. The five candidate gene polymorphisms investigated in this study were *IL-6* (rs1800796; -572G/C), *IL-8* (rs4073; -251 T/A), *ACE* (rs4646994; I/D), *VEGF* (rs3025039 and rs2010963; +936 C/G and +405 G/C respectively); and *AGT* (rs699; +842 G/A) which were selected based on the crucial role they play in renal function.

In a previous study, researchers reported that low levels of haemoglobin may lead to hypoxia which causes inadequate oxygen delivery to the tissues (Celsing *et al.,* 1987). In addition, other studies reported that exposure to contrast media cause increased haemoglobin-oxygen affinity, which aggravates hypoxic injury and further results in renal dysfunction thus making patients with decreased levels of haemoglobin at a higher risk of developing CIN (Cigarroa *et al.,* 1989; Kim *et al.,* 1990; Heyman *et al.,* 2012). Similarly, when examining the baseline data, the present study demonstrated lower levels of haemoglobin in CIN+ patients compared to patients without. The results illustrated an association between haemoglobin and CIN ($p = 0.006$). Our finding is similar to a retrospective study conducted by Xu and colleagues, who reported a 3-fold increase in the prevalence of CIN development in patients with low levels of haemoglobin compared to patients with normal levels (Xu *et al*., 2016). A second study observed that a decrease in haematocrit caused higher levels of CIN prevalence in patients with or without CKD, $p<0.01$ (Nikolsky *et al*., 2005).

In our study, the mortality rate was significantly increased in the patients with CIN compared to the CIN negative patients (24.1% versus 7.5%; $p=0.007$). Our findings are similar to previous studies that demonstrated higher mortality rates among CIN+ patients than CIN- patients. In these studies, CIN caused a 7-22% or more in-hospital mortality (Levy *et al.,* 1996; McCullough *et al*., 1997; Rihal *et al*., 2002). According to Levy *et al.* (1996), the mortality rate in white patients with renal failure was 34% compared with 7% of those without renal failure undergoing radiocontrast

procedures (p<0.001). In another study of patients undergoing coronary intervention at William Beaumont Hospital in the United States, investigators reported a 35.7% in-hospital mortality for those who developed acute renal failure requiring dialysis (McCullough *et al*., 1997). Additionally, Rihal *et al.* (2002) reported a 22% in-hospital mortality among patients with acute renal failure (ARF) compared with only 1.4% of patients without ARF after PCI in the United States (p<0.0001).

Inflammatory *IL-6*, *IL-8*, *ACE*, *VEGF* and *AGT* polymorphisms were associated with the development of AKI in Caucasians, Southeast Asians, children, and patients with added comorbidities including severe sepsis following radiological procedures (Stafford-Smith *et al.*, 2005; Alge *et al*., 2013; Cardinal-Fernández *et al.*, 2013; Greenberg *et al*., 2015; de Fontnouvelle *et al.*, 2017; Saw *et al*., 2019). However, none of these variants have been associated with kidney disease in a black population. Thus, to our knowledge this is the first study to report no evidence of an association between cytokine inflammatory polymorphisms in the *IL-6, IL-8, ACE, VEGF*, and *AGT* genes with the development of CIN in a black South African population.

The statistical analysis included the four genetic models (dominant, recessive, multiplicative and additive) which were used to assess the association between polymorphisms in candidate genes and CIN. Allelic and genotypic tests were the two approaches used in the genetic models to test the association. In this study, an increase in the frequency of the minor alleles observed in these genetic models could also cause an increase in the risk of CIN.

The dominant genetic model for each of the SNPs was analysed using homozygous major alleles and treating the heterozygote (minor and major alleles) and minor allele homozygote genotypes as a single category, thus heterozygous and minor alleles (homozygote genotype) modify disease risk. Alternatively, a recessive model was used with minor alleles (homozygote) and two genotypes (heterozygote and dominant homozygote) treated as one entity, therefore the minor homozygote was related to disease risk. The third genetic model used was the multiplicative model, analysed using allele frequency where disease risk increased by a factor r for each minor allele carried (r-fold increased risk for heterozygote and r^2 increased risk for homozygote minor genotype). The fourth genetic model used was the additive model, with an increase in disease risk of r for heterozygote genotypes, and a 2r increase for the minor allele (homozygote genotype). Therefore, this model illustrates that an increase in heterozygote and homozygote minor allele

genotypes increases risk of disease, by 2-fold for the homozygous minor allele and 1-fold for the heterozygote genotypes (Lewis, 2002).

4.1. Association with *IL-6*

Interleukin-6 (IL-6) is one of the main proinflammatory cytokines which play a significant role in the induction of AKI via two methods: A cytokine-dependent cell mediated immune response that increases renal damage and by having a protective role in tubular epithelial cells that reduces injury thus maintaining renal function (Nechemia-Arbely *et al*., 2008). In addition, Nechemia-Arbely *et al*. reported that *IL-6* is important to the inflammatory response of renal injury. In a recent study, Saw *et al*. (2019) demonstrated that the *IL-6*-572C allele was positively associated with a reduced risk of AKI (AOR: 0.57) and that this polymorphism had a protective role in AKI (83.6% vs 91.2%, $p<0.001$).

In our findings, the *IL-6* polymorphism (rs1800796) was not statistically associated with CIN. The allelic and genotypic distribution among CIN positive patients and those without CIN were similar, with all *p*-values greater than the level of significance ($\alpha = 0.05$). The dominant genetic model (GG versus GC+CC) and the recessive model (CC versus GC+GG) both revealed no significant association with risk of CIN ($p= 0.591$ and $p= 1.000$, respectively). Additionally, the additive genetic model (GC versus CC versus GG) also demonstrated no association with CIN risk among black South Africans (β- Coef: 0.016; 95% CI: -0.0909825, 0.123; $p= 0.769$). These results are similar to those reported by Al-Radeef *et al*. in Iraq which found a similar distribution of *IL-6* genotypic ($p= 0.467$) and allelic ($p= 0.322$) frequencies between patients with chronic renal failure and those without, with the GG genotype being the most frequent followed by the GC and CC genotypes (Al-Radeef *et al*., 2018). In another study in Western Europe, Losito and colleagues also documented similar genotypic frequencies between patients with end-stage renal disease (ESRD) and those without (Losito *et al*., 2003). In this study investigators observed the frequency of GG, GC, and CC to be 53.4%, 32.4%, and 6.2%, respectively in patients with ESRD and 59.7%, 34.3%, and 3.5%, respectively in patients without ESRD (Losito *et al*., 2003).

This present study is in contrast with a study conducted among Caucasians which demonstrated a significant association between the *IL*-6-572C allele and the risk of AKI, $p < 0.0001$ (Stafford-Smith *et al*., 2005). In a prospective study of patients who underwent cardiac surgery, Zhang *et al*. also reported increased risk of AKI in patients carrying elevated levels of IL-6 post cardiac surgery

and that mortality rates were associated with high concentrations of IL-10, p= 0.01 (Zhang *et al*., 2015). These findings suggest that elevated levels of IL-6 and IL-10 serve as biomarkers for outcomes following cardiac surgery in their population (Zhang *et al*., 2015). In another study of children undergoing cardiopulmonary bypass (CPB), investigators reported significantly higher levels of preoperative IL-6 in patients with stage 2/3 AKI than in those without (Greenberg *et al*., 2015).

4.2. Association with *IL-8*

Interleukin-8 (IL-8) is a pro-inflammatory chemokine produced by multiple cells including lymphocytes and mast cells upon exposure to the inflammatory milieu (Baggiolini *et al*., 1989). The release of IL-8 leads to the activation and migration of neutrophils to the site of infection which causes clearance of pathogens (Benakanakere *et al*., 2016). Increased levels of IL-8 lead to inflammation in chronic inflammatory diseases and inhibition of this chemokine may delay the influx of neutrophils, resulting in pathogen survival which can cause chronic infections (Ferreira Jr *et al*., 2008; Kinane *et al*., 2008; Benakanakere *et al*., 2016). Previous studies reported an association between elevated levels of IL-8 and several diseases including chronic periodontitis, carcinogenesis, chronic obstructive pulmonary disease, and hypertension (Konopka *et al*., 2012; Gales *et al*., 2013; Caramori *et al*., 2014; Gilowska, 2014; Martynowicz *et al*., 2014).

In this study, we found no association between the *IL-8* polymorphism (rs4073) and the risk of CIN development. Both the allelic and genotypic frequencies among the CIN positive group and the non CIN group were similar which indicated no association (all p>0.05). The recessive model (TT versus AT+AA) showed no association between the rs4073 SNP and CIN (p= 1.000). Similarly, the dominant model (AA versus AT+TT) showed no link between the *IL-8* polymorphism and the risk of CIN development (p= 0.103). Furthermore, the additive model (AT, TT versus AA) also resulted in no significant association with the risk of CIN with β- coeff of 0.055 (95% CI= -0.036862: 0.148) (p= 0.238). Our findings contrast with several studies that have found a link between this *IL-8* polymorphism and other diseases including AKI following liver transplants, radiological and cardiovascular procedures. According to McBride and co-workers, cardiac surgery caused elevated levels of pro-inflammatory mediators in plasma including IL-8 (McBride *et al*., 1995).

In a previous prospective study of 143 patients who underwent on-pump cardiac surgery, Liangos *et al*. observed an increase in IL-8 plasma levels among patients who developed AKI compared to those without AKI following cardiac surgery (Liangos *et al*., 2009). They concluded that IL-8 levels in plasma predict the risk of developing AKI (Liangos *et al*., 2009). Similarly in another study, serum levels of IL-8 were elevated in patients with AKI compared to those without post liver transplantation (p= 0.0463) with IL-8 as one of the biomarkers of AKI (Sirota *et al.,* 2013). Cardinal-Fernández *et al*. also found a significant association between the polymorphism *IL-8* 251 AA and an elevated risk of AKI (OR= 4.98; 95% CI= 1.04-23.86), p= 0.045 (Cardinal-Fernández *et al*., 2013).

In a study of children undergoing cardiac surgery, Liu and colleagues reported that patients with AKI had significantly higher levels of serum IL-8 compared to those without AKI and further suggested that IL-8 is associated with AKI development in a pediatric population post cardiac surgery (Liu *et al*., 2009). But in this study the association was modest and had a small sample size (Liu *et al*., 2009). Furthermore, investigators have demonstrated that increased levels of IL-8 are associated with higher odds of AKI in large cohorts of adult patients with various inflammatory conditions (De Pablo *et al.,* 2013; Liu *et al.,* 2014).

4.3. Association with *ACE*

In our study, we did not observe any differences between genotypic or allelic frequencies of the *ACE* SNP rs4646994 for patients with CIN compared to those without CIN (all p >0.05). The dominant model (DD versus ID+II) of the *ACE* (rs4646994) SNP revealed no association with risk of CIN development (p= 0.939), and the recessive model (II versus ID+DD) showed no association with CIN (p= 0.651). Also, the additive model (ID, II versus DD) illustrated no association with risk of CIN indicated by β- coeff (95% CI): 0.011 (-0.0686398; 0.091), p= 0.780. This contrasts with other studies that found an association (Stafford-Smith *et al*., 2005; Isbir *et al*., 2007; du Cheyron *et al.,* 2008; He *et al*., 2013; Saw *et al*., 2019).

In a previous study by Stafford-Smith and colleagues, the presence of the *ACE*-D allele was associated with risk of AKI among African Americans after cardiac surgery (Stafford-Smith *et al*., 2005). Isbir and co-workers also reported a significant association between the *ACE*-D allele (p= 0.018) and the development of AKI following cardiac surgery (Isbir *et al*., 2007). In a study among the Chinese population, He *et al*. reported that the *ACE*-D allele was positively associated with

hypertension. These investigators observed an adjusted OR of 1.94 (95% CI: 1.01-3.73) for the *ACE* DD genotype indicating a role for the *ACE*-D allele and/or DD genotype as a risk factor (He *et al*., 2013). A recent study among a Southeast Asian population demonstrated a positive association between the *ACE*-D allele (64.1% vs 55.6%, $p<0.001$) and an increased risk of AKI (Saw *et al*., 2019). However, in a previous study of critically ill white patients admitted to the intensive care unit (ICU), Cheyron *et al*. demonstrated the *ACE*-I genetic polymorphism (*ACE* II genotype) as a risk factor in AKI development and outcome (du Cheyron *et al.,* 2008).

Our findings are similar to a previous study conducted among southern Brazilians (Pedroso *et al*., 2010). These investigators also illustrated no significant association between genotypic or allelic frequencies and renal dysfunction (Pedroso *et al*., 2010). Cardinal-Fernández *et al*. (2013) also reported no association between the *ACE* II genotype and risk of AKI among white patients admitted to the ICU. In another study in the United Kingdom, investigators found no significant association of the *ACE*-D allele with risk of AKI (McBride *et al*., 2013).

4.4. Association with *VEGF*

The vascular endothelial growth factor (VEGF) is a pro-inflammatory cytokine promoting proliferation, differentiation, and survival of endothelial cells (Schrijvers *et al.,* 2004). Elevated levels of VEGF are found in glomerular podocytes and tubular epithelial cells, while VEGF receptors are predominantly found on preglomerular, glomerular, and peritubular endothelial cells (Schrijvers *et al.,* 2004). In acute renal failure (ARF), hypoxia is a potent stimulus that causes the production of VEGF in some cells (Minchenko *et al*., 1994). In another study, investigators observed elevated levels of VEGF following hypoxic injury in rat glomerular epithelial cells and human renal proximal tubular epithelial cells in culture (Kim *et al*., 2002). The outer medulla region of the kidney is more susceptible to hypoxia which induces inflammatory cytokine generation including VEGF. Thus, VEGF plays a critical role in the pathophysiology of renal disease including CIN (Epstein *et al*., 1994, Fine *et al*., 1998).

In the present study, we investigated two polymorphisms, rs3025039 (+936 C/G) and rs2010963 (+405 G/C) located in the *VEGF* candidate gene. In our findings, both SNPs rs3025039 and rs2010963 demonstrated no association with CIN. The allelic and genotypic frequencies for both SNPs were similar between CIN+ patients and those without CIN (the control group). The dominant genetic model (CC versus CT+TT) and the recessive genetic model (TT versus CT+CC)

of SNP rs3025039 revealed no association with risk of CIN with *p*-values all above 0.05 (*p*= 0.386 and *p*= 1.000 respectively). The additive model (CT, TT versus CC) also indicated no significant association with risk of CIN development, *β*- Coef (95% CI): -0.083 (-0.2179636; 0.051) (*p*= 0.238). For SNP rs2010963, both the recessive (CC versus GC+GG) and dominant genetic models (GG versus GC+CC) showed no association with CIN (*p*= 0.359 and *p*= 0.740, respectively). The additive model also confirmed no association with risk of CIN development with a *β*- coeff of 0.006 and 95% CI: (-0.0624888; 0.076), *p*= 0.851. Similar to our finding, Cardinal-Fernández and co-workers also found no association between *VEGF* + 405 CC genotype (rs2010963 SNP) and risk of AKI with OR (95% CI): 1.33 (0.48-3.70), *p*= 0.58 (Cardinal-Fernández *et al.*, 2013).

These results contrast with studies that found a significant association between the *VEGF* polymorphisms (rs3025039 and rs2010963) and acute renal insufficiency and other diseases (Bányász *et al*., 2006; Cardinal-Fernández *et al.*, 2013; Ma *et al*., 2018). In a study of critically ill patients with severe sepsis, Cardinal-Fernández and colleagues reported that the *VEGF* + 936 CC genotype is statistically associated with an elevated risk of AKI development (*p*= 0.036) in a univariate analysis (Cardinal-Fernández *et al.*, 2013). However, they did not find statistical significance with the *VEGF* + 936 CC genotype in a multivariate analysis (OR, 4.23; 0.78-22.82; *p*= 0.094), probably because of the limited statistical power of the study (Cardinal-Fernández *et al*., 2013).

A meta-analysis by Hou and colleagues found seven studies illustrating a positive association between the rs2010963 (GG and GC+CC genotypes) polymorphism and increased risk of renal cell carcinoma. Hou *et al*. further revealed that the CC and CC+CT genotype of the *VEGF* rs3025039 polymorphism were associated with lower odds of renal cell carcinoma when compared to the TT genotype (Hou *et al*., 2017). Gong *et al*. (2017) indicated that *VEGF* +936C/T genotype was associated with an elevated risk for renal cell carcinoma. A meta-analysis by Gong *et al*. found no association between -405G/C (rs2010963) and renal cell carcinoma (Gong *et al*., 2017).

4.5. Association with *AGT*

Angiotensinogen is an inactive precursor molecule of angiotensin (Ang II), and its synthesis is stimulated by hormones in the body including estrogen and Ang II (Kaschina *et al*., 2018). Elevated levels of angiotensinogen are significantly associated with hypertension and elevated Ang II levels and results in the production of ROS and alterations in renal heamodynamics, both

mechanisms associated with CIN (Kaschina *et al*., 2018; Satou *et al*., 2018). Ang II is a growth factor that regulates cell proliferation and apoptosis (Wolf & Neilson, 1993; Ruiz-Ortega *et al*., 2001). In addition, Ang II functions as a proinflammatory mediator, participating in the inflammatory response of pathological mechanisms associated with types of AKI including CIN (Ruiz-Ortega *et al*., 2001).

In this present study, the *AGT* rs699 polymorphism was not significantly associated with risk of CIN, with *p*-values greater than 0.05 in all the genetic models. The dominant genetic model (GG versus GA+AA) of *AGT* rs699 genotype revealed no association with CIN (p= 0.989). The recessive model (AA versus GA+GG; p= 0.598) and additive model (GA, AA versus GG: β- Coef= -0.017, 95% CI= -0.1148853; 0.080, p= 0.724) also demonstrated no association with the risk of developing CIN in the black population. Several studies contrast with our current study findings. In a previous study conducted among Caucasian patients, Stafford-Smith *et al.* reported that the *AGT* variant (*AGT* +842 C genotype) was associated with increased serum creatinine levels of 121% ($p < 0.0001$) and that the combination of *AGT* and *IL-6* polymorphisms (*AGT* +842C and *IL-6* 572-C, respectively) was associated with renal dysfunction (Stafford-Smith *et al.*, 2005).

In other studies, this polymorphism in *AGT* was linked to the development of hypertension and possibly of kidney disease in French and Japanese populations (Jeunemaitre *et al*., 1992; Hata *et al*., 1994). However, these findings were not supported in further association studies. Investigators found a non-significant association between the *AGT* polymorphism and the development of hypertension in the African American, European, and Japanese populations (Rotimi *et al*., 1994; Brand *et al*., 1998; Kato *et al*., 1999). A different *AGT* polymorphism (M268T) was reported as being significantly associated with the development of nephropathy in patients with diabetes mellitus in a Pakistani population (Shaikh *et al*., 2014). On the contrary, our findings are similar to a study of Finnish ICU patients, where Vilander *et al*. were unable to demonstrate any significant association between the *AGT* genetic variant (rs699) and AKI in critically ill patients (Vilander *et al*., 2019).

In summary, we investigated the role that inflammatory and vasoconstrictor gene polymorphisms play in CIN development following contrast administration in a black South African population. All the gene (*IL-6*, *IL-8*, *ACE*, *VEGF* and *AGT*) polymorphisms had no significant link to risk of CIN development. However, in a study conducted among black South Africans, Banda confirmed

the association of the inflammatory *TNFα* 308 AA (high producer) genotype with susceptibility to CIN development while the *IL-10* -1082 AA (low producer) SNP genotype caused a decline in survival among patients with CIN (Banda, 2017). Similarly, in a study among the Chinese Han population, Chang *et al*. demonstrated association of inflammatory *IL-10* and *TNF-α* gene polymorphisms with CIN development following PCI (Chang *et al*., 2013). These findings support mechanisms that may contribute to renal injury.

LIMITATIONS OF THE STUDY

Although this is the first study that focused on the association of *IL-6, IL-8, ACE, VEGF* and *AGT* genetic polymorphisms with risk of CIN among black South Africans, the study had some limitations. Firstly, samples were collected from a single tertiary hospital thus may not be a representative of all black South Africans. Secondly, our results lacked sufficient statistical power to assess the association between genetic variants in the *IL-6*, *IL-8*, *ACE*, *VEGF* and *AGT* genes and susceptibility to CIN due to the small sample size. Our study did not reach statistical significance because of a lack of statistical power and the small sample size of 30 CIN patients compared to 198 non-CIN patients. Given the small minor allele frequencies of the SNPs examined, the study was underpowered to detect associations at low odds ratios. Thus, although associations between these SNPs and CIN may exist in the black South African population, we were unable to detect them. Furthermore, serum levels of IL-6, IL-8, ACE, VEGF and AGT genes were not measured but will form part of future studies in this cohort.

CONCLUSION

In conclusion, this retrospective study shows that frequencies of all the gene variants including rs1800796, rs4073, rs4646994, rs3025039, rs2010963, and rs699 SNPs did not differ between CIN patients and control patients after contrast administration. However, elevated mortality rates were associated with CIN+ patients compared to the control group (CIN-). Further studies with larger sample sizes of CIN+ patients are needed to provide a more robust statistical analysis.

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Appendices

APPENDIX A

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms Nomthandazo Twala et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190692 Sub-Study

NAME: (Principal Investigator)	Ms Nomthandazo Twala et al
DEPARTMENT:	Internal Medicine
PROJECT TITLE:	Genetic polymorphism of angiotensin-converting enzyme (ACE), Interleukin-6 (IL-6), Interleukin-8 (IL-8), vascular endothelial growth factor (VEGF) and angiotensinogen (AGT) associated with contrast induced nephropathy in black South Africans
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub Study under primary study M190762 (previously M140467) Dr Justor Banda
SUPERVISOR:	Dr Caroline Dickens and Dr Justor Banda
APPROVED BY:	Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	29/08/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed June and will therefore be due in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Dr J Banda
School of Clinical Medicine
Department of Medicine
Division of Internal Medicine
Charlotte Maxeke Johannesburg Academic Hospital

E-mail: katusib@yahoo.co.uk

CC: Supervisor: Not applicable <>
and <HREC-Medical ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2019/08/12

REF: *R14/49*

PROTOCOL NO: **M190762** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Biomarkers in acute kidney injury due to contrast-induced nephropathy*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

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UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr J Banda

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190762

NAME: (Principal Investigator)	Dr J Banda
DEPARTMENT:	School of Clinical Medicine Department of Medicine Division of Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Biomarkers in acute kidney injury due to contrast-induced nephropathy
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal of M140467
SUPERVISOR:	Not applicable
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2019/08/12

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **July** and will therefore reports and re-certification will be due early in the month of **July** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES

APPENDIX C

REPUBLIC OF ZAMBIA

All correspondence should be address
Senior Medical Superintendent
Ndola Teaching Hospital
Postal Agency
NDOLA

Telephone: 611585-9
Fax: 612204
E-mail: *ndolateachinghospital@gmail.com*

MINISTRY OF HEALTH
NDOLA TEACHING

June 5, 2019

Nomthandazo Twala
Wits University
South Africa

Dear Nomthandazo

REF: REQUEST FOR PERMISSION TO USE WHOLE BLOOD SAMPLES AND DATA

Reference is made to your letter dated June 5, 2019 in which you are seeking authority to use my study samples at Charlotte Maxeke Johannesburg Academic Hospital, in South Africa for the purpose of your MSc study under the supervision of Dr Caroline Dickens and myself.

Permission is granted for you to undertake a sub study using my samples that I collected during my PhD studies (ethics clearance no. M140467) for your study entitled "Genetic polymorphisms of ACE, IL6, IL8, VEGF and AGT association with contrast induced nephropathy"

Yours sincerely
NDOLA TEACHING HOSPITAL

Dr Justor Banda PhD (Wits), FCP (ECSA), MMed, MBChB
Head of Department- nephrology
For/ Senior Medical Superintendent

APPENDIX D

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APPENDIX E

Data collection sheet

Identification number: _____

Date: _____ _____/_____

Title: **Genetic polymorphisms of *ACE, IL-6, IL-8, VEGF* and *AGT* associated with contrast induced nephropathy in black South Africans.**

1. **Demographic data**

   a. Age ___ ____

   b. Gender: □ male □ female

   c. Race: □Black □Caucasian □ mixed race □Indian

2. **History**

   a. History of kidney disease: □Y □N if yes specify_____

   b. DM: □ Y □N, if yes

      a. duration of DM ;_____

      b. DM medications _____

   c. Hypertension □Y □N_____

      a. Duration of HTN

      b. HTN medications

   d. Medications :

      a. Medications: ACEI_____

      b. Diuretics_____

      c. Aminoglycosides _____

      d. NAC_____

      e. NSAIDS _____

      f. Other medications; specify_____

e. HIV status: □+ □- □unknown, if reactive CD4:_____ VL_____

i. HAART: [ ]+ [ ]- type_____

f. Type of contrast media dose_____

a. Dose of contrast media(ml)

b. Location of contrast(IV/IA) _____

c. Type of contrast medium _____

g. In patient diagnosis 1_____

a. Diagnosis 2_____

h. Pre existing co-morbidities: [ ]Cardiac disease [ ]anemia [ ] malignancy

[ ] Stroke [ ] others

3. Clinical parameters

a. BP:_____/_____ MAP_____ RR_____ Temp___/_____

b. HR ___ GCS_____ BMI_____

c. Pre contrast fluid status:

a. volume/hour_____

b. Type of fluid_____

c. Urine output mls_____

d. Total volume before contrast study_____

d. Post contrast fluid status

a. Volume_____

b. Type of fluid_____

c. Urine output mls/hr_____

Base characteristics

Serum creatine umol/l		
Serum urea mmol/l		
eGFR		
Genotypes		
ACE		
IL-6 (rs1800796)		
IL-8 (rs4073)		
VEGF (rs3025039)		
AGT (rs699)		
CMP/PTH		
NA		
K		
CO		
CI		
FBC		
Albumin		
TC		
TG		
LDL		
HDL		
Imaging parameters	**CT scan**	**Echocardiography**
LVEDD (echo only)		
LVSD (echo only		
IVC (CT& echo)		

Post contrast media measurements

Serum creatinine			
Serum urea			
eGFR			

APPENDIX F

Maxwell® 16 Blood DNA Purification Kit (Promega AS1010, Madison, Wisconsin, USA)

Materials required for 48 automated isolations from 50-400 µl whole blood samples include:

• 48 Maxwell® 16 Blood DNA Cartridges
• 50 Purification Plungers
• 50 Elution Tubes
• 20 ml Elution Buffer

Protocol:

- Whole blood samples collected in EDTA tubes were used. These samples may be either fresh or frozen. Frozen samples were thawed and mixed before processing.
- Add 300 µl of elution buffer to each blue elution tube and place the tubes in the correct slots at the front of the platform.
- Cartridges are placed into the holder in the correct orientation and the seal removed from each cartridge.
- A plunger is placed into well #7 of each cartridge
- Up to 400 µl of whole blood is added to well #1
- The Maxwell® 16 Instrument is turned on, checked that the SEV setting is selected and that the SEV hardware is present.
- The protocol for blood DNA extraction is selected and the instrument set to run.
- The concentration of DNA was measured by the NanoDrop™ 2000 spectrophotometer and stored at -20 °C.

APPENDIX G

SALTING OUT METHOD (modified from Miller *et al*., 1988)

Solutions Needed:

1. **Sucrose-Triton-X Lysis Buffer**
   - Add 10 ml Tris-HCL pH 8.0, 5 ml of 1M $MgCl_2$, 10 ml Triton X-100, make up to 1 litre with distilled water.
   - Autoclave and store at 4 ^oC.
   - Add 109.5 g sucrose-**just before use.**
2. **T20E5 (20 mM TRIS-HCl, 5 mM EDTA)**
   - Add 20 ml 1M Tris-HCl, 10 ml 0.5M EDTA, make up to 1 litre with distilled water.
   - pH 8.0
   - Autoclave and store at room temperature.
3. **Saturated Salt (NaCl) Solution**
   - Slowly add 40 g NaCl to 100 ml sterile water until completely saturated.
   - Filter-sterilize and store at room temperature.
4. **Proteinase K-Master Mix**
   - For 16 extractions; add 800 µl 10% SDS, 32 µl 0.5 M EDTA, 6 400 µl distilled water.
   - Add 800 µl Proteinase-K (20 mg/ml stock)-**Just before use**.
5. **TE buffer (10 mM Tris-HCL, 1 mM EDTA)**
   - Add 10 ml 1M Tris-HCL (pH 7.5), 2 ml 0.5M EDTA pH 8, and make up to 1 litre with distilled water.
   - Autoclave.

Procedure

Day 1

1. Pre-cool the centrifuge to 4 °C.
2. Blood must be collected into EDTA tubes.
3. Use ±10 ml blood, place in a 50 ml tube and mix with ±40 ml lysing solution.
   - NB: Lysing solution must be kept cold during the procedure. Mix well. Work on ice.
4. Spin for 10 min at ± 2300 rpm (setting 5 on the Sorvall Centrifuge) at 4 ^oC.
5. Decant supernatant and re-suspend pellet in ± 20 ml lysing buffer. Work on ice.
6. Place on ice or at –20 ^oC for 5 min.
7. Spin for a further 10 min at 2300 rpm (setting 5) at 4 ^oC.
8. Decant supernatant and add 3 ml T20E5 to each tube. Mix well.
9. Add 200 µl of 10% SDS to each and mix.
   - Then, add 495 µl proteinase K mix, mix and place samples at 42 ^oC- 50 ^oC overnight.

Day 2

10. Add 1 ml saturated NaCl solution to each tube. Mix well and place on ice for 5 min.
11. Spin at ± 2800 rpm (setting 5 ½ on the Sorvall Centrifuge) for 30 min, 4 °C.

- If the supernatant is cloudy, decant into a clean tube and spin for a further 15- 20 min. The pellet consists of proteins precipitated by the salt and the DNA should be present in the supernatant.

12. Transfer the supernatant to a clean 50 ml tube and add 20 ml absolute ethanol at room temperature.
13. DNA should be visible as a white aggregate.
    - **If none is visible**, place samples at *– 20 ºC overnight, or at -70°C for 3 hours. (See 13b below).
14. Otherwise, fish or spool the DNA out using a P200 Gilson, wash gently in ice cold 70% ethanol (approx. 200 µl). Do this twice.
15. Place in a clean Eppendorf tube and allow ethanol to evaporate off at room temperature. Do not allow to over dry.
16. Dissolve DNA in an appropriate volume (~100 µl) of TE buffer to a maximum of 1 ml. Put at 37 ºC to dissolve completely (can leave at 37 °C/room temperature overnight to ensure DNA has dissolved.
17. Check DNA concentration on Nanodrop. Mix well before measuring concentration.

*__Day 3__ (if DNA samples did not aggregate)

13b. Centrifuge samples in absolute ethanol at 2800 rpm (setting 5 ½ on the Sorvall Centrifuge), 4 °C, 15 mins

13c. Pour off absolute alcohol. Add 1 ml of ice-cold 70% ethanol.

13d. Centrifuge at 2800 rpm, 4 °C, 15 mins.

13e. Remove 70% Ethanol and wash a second time with 1 ml ice-cold 70% ethanol.

13f. Centrifuge at 2800 rpm, 4 °C, 15 mins. Remove ethanol (Blot excess ethanol on tissue paper). Allow pellet to dry (but not over dry).

13h. Add 100 µl TE buffer. Close lid. Allow DNA pellet to dissolve in TE overnight, RT°, shaker.

13i. Transfer to a labelled 1.5 ml Eppendorf.

13j. Measure DNA concentration.

APPENDIX H

REAGENTS

10X Tris-Boric Acid-EDTA (TBE) buffer

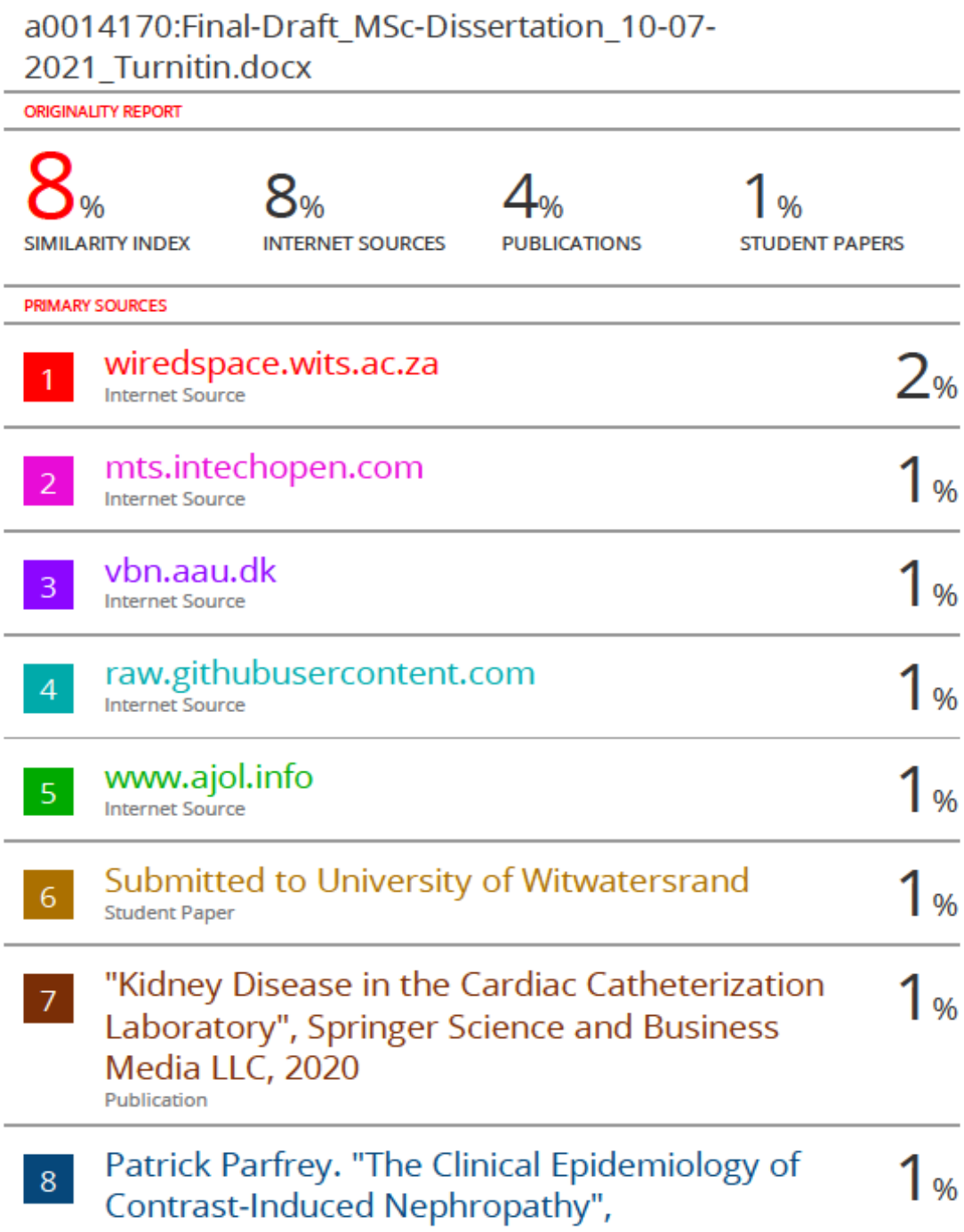
- 54 g Tris (ICN Biomedicals, Ohio)
- 27.5 g Boric acid
- g EDTA (Sigma-Aldrich, Germany)
- Add distilled water and make up to a final volume of 500 ml
- Autoclave before use

1X TBE buffer

- 100 ml of 10X TBE buffer
- Make up to 1000 ml with distilled water

APPENDIX I

Turnitin Report



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