TYPE 2 DIABETES MELLITUS AND DYSLIPIDAEMIA: EFFECTS OF GENETIC VARIATION IN AFRICAN POPULATIONS

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I, Donald Moshen Tanyanyiwa, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been submitted for another degree in this or any other university.

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ABSTRACT

Background: Low-density lipoproteins (LDL) have been associated with damage to the cardiovascular system in patients with type 2 diabetes mellitus (T2DM). These patients are two (2X) to four (4X) times more likely to develop cardiovascular diseases (CVD) compared to non-diabetic patients due to dysfunctional lipoprotein metabolism. Normal lipid metabolism involves interconversion and transfer of molecules regulated by several enzymes such as Apolipoprotein E (ApoE) and proprotein convertase subtilisin/kexin type 9 (PCSK9). ApoE and PCSK9 are involved in clearance of lipoproteins and therefore, influence lipid profiles. Association between ApoE and T2DM in cardiovascular diseases have been widely reported. PCSK9 on the other hand is emerging as an important player in lipid metabolism but its effects in diabetes are not known. Studies on both ApoE and PCSK9 in African populations are in its infancy. Each year, CVD kills more people than any other cause of death. Many CVDs can be traced back to pathological process of atherosclerosis, in which fatty material collects along walls of arteries, limiting flexibility and obstructing blood flow. T2DM alone has been classified as a major factor for development of CVD and one of its complications is the development of dyslipidaemia. Unlike PCSK9, genetic polymorphisms in ApoE have been well characterised as important dyslipidaemia genetic markers associated with coronary artery disease. The association between ApoE and PCSK9 gene polymorphisms with dyslipidaemia in T2DM was evaluated. Diabetic dyslipidaemia presents as a triad of high triglycerides, high LDL and low high-density lipoprotein (HDL).

Aims and Objectives: This study aimed to evaluate the role of genetic variation in genes coding for ApoE2 and PCSK9 on dyslipidaemia in South African diabetic patients. Main objectives' included recruitment of participants, genetic characterisation of ApoE and PCSK9 and determination of the lipid profiles for the recruited participants.

Methods: Two hundred and forty-four (n=244) participants were recruited from the Baragwaneth diabetic clinic, using a retrospective approach. The participants comprised of two groups, (i) dyslipidaemic, and (ii) non-dyslipidaemic (controls). The dyslipidaemic group was further divided into three groups; i) those with high cholesterol only, ii) those with high triglycerides only and iii) those with both high cholesterol and triglycerides which is referred to as the mixed group. Clinical and demographic parameters were retrieved from hospital records with the consent of the participants. Ethical clearance was obtained from the

University of Cape Town and University of Witwatersrand. Genetic characterisation of ApoE was carried out using polymerase chain reaction (PCR) coupled to restriction fragment length polymorphism (RFLP) and confirmed through sequencing while characterisation for PCSK9 was carried out through Sanger sequencing.

Results: Of the 244 participants, 165 were dyslipidaemic while 79 were not dyslipidaemic. The 165 dyslipidaemic participants were further divided into 33.3% (n=55) those with high cholesterol, 29.1% (n=48) those with high triglycerides and 37.6% and (n= 62) those with high cholesterol and triglycerides (mixed). The cohort comprised of 128 (52%) females, median (IQR) age 56.0 (48.0 – 64.0) years and 116 (48%) males with median (IQR) age of 56.5 (48.0 – 63.0) years. Most of the characteristics between the dyslipidaemic and non-dyslipidaemic participants were significantly different as expected in a purposive sampling technique. ApoE3/4 genotype had the highest frequency distribution (41%) while ApoE2/3 genotype had the lowest frequency (7%). An uncharacterised ApoE referred to in the study as ApoE X with a frequency distribution of 6%, was reported for the first time. The selected measured parameters evaluated against a set of variables showed a significant association between HbA1c and age (p=<0.008) is reported. TC (p=0.00092), LDL (p=0.0184) and TG (p=0.0175) were strongly associated with poor glycaemic. Both LDL (p=0.0174 and HDL (p=0.0072) were associated with age. Homozygous *ApoE2/2* and heterozygous *ApoE2/3* genotypes correlated with poor glycaemic control with a median HbA1c of 10.95% (IQR 5.88-14.98%) and 10.20% (IQR 6.20-15.80%), respectively; while homozygous ApoE4/4 carriers displayed good glycaemic control with a median HbA1c of 6.60% (IQR 5.70 – 2.30%). Carriers of homozygous *ApoE3/3* genotype had the highest median TC of 6.06mmol/L (IQR 5.48 -– 6.71mmol/L) while homozygous *ApoE4/4* carriers had the highest median triglycerides of (2.94 (IQR 1.75 – 5.13 mmol/L). Carriers of homozygous *PCSK9 rs505151 A/A* (E670G) genotype had the highest frequency distribution in both groups of participants with dyslipidaemic (55.1%) and non-dyslipidaemic (63. 5%), followed by carriers of heterozygous PCSK9 rs505151G/A at 40.6% and lastly carriers of *PCSK9 rs505151G/G* at (9.5%). On the other hand, carriers of homozygous *PCSK9 rs28362286 C/C* genotype were predominantly distributed with a frequency of 94.2% and *PCSK9rs28362286C/A* had a very small frequency distribution of 5.8% while *PCSK9rs28362286A/A* was absent in this population. Carriers of *PCSK9 rs505151A/A* genotype had higher HbA1c with a median of 10.10% (IQR 7.48 – 12.90) compared to

PCSK9 rs505151 G/A genotype with a median of 9.00% (IQR 7.03 –11.35). The results show that *PCSK9 rs505151G/A* with lower HbA1c had non-significantly higher TC, LDL, TG and non-HDL but lower HDL compared to *PCSK9 rs505151A/A* genotype. The results revealed no direct reciprocal relationship between glycaemic control and level or type of dyslipidaemia.

Conclusions: The study showed the effects of ApoE and PCSK9 genetic variation on the dyslipidaemia seen in black South African diabetic participants. Therefore, this study through ApoE and PCSK9 genotypes show that the diabetic dyslipidaemia has an underlying genetic influence. In addition, to the well-characterised ApoE genotypes, an uncharacterised genotype referred to as ApoE X genotype is reported. With these findings, consideration to explore possible underlying genetic predisposition is recommended especially in diabetic patients with dyslipidaemia that responds poorly to standard therapy.

PREFACE

This thesis is being submitted in accordance with general provision 6.9 in the General Rules for the degree of Doctor of Philosophy (PhD) of the University of Cape Town. **Chapter 1** is a general literature review looking at the aspects directly and indirectly related to the thesis. **Chapters 2 to 4** are specific to the subject, which include methods, results and discussion of results.

The thesis outline is as follows:

Chapter 1: This is a general introduction encompassing background on the aims and objectives. The main aims and objectives are covered in section 1.6.

Chapter 2: This chapter presents the methods and materials, from the study population, laboratory routine analytical methods for lipids, lipoproteins, HbA1c, isolation, and purification of DNA as well as genotyping of ApoE.

Chapter 3: This chapter presents the demographic characteristics of the cohort, Apolipoprotein E and PCSK9 genotype frequency and the associated pattern of dyslipidaemia

Chapter 4: This chapter presents discussions on all the findings from the genetic characterisation of ApoE and PCSK9 forms dyslipidaemia.

Chapter 5: Conclusion, Strengths', Weaknesses and Recommendations.

References: The section acknowledges all work quoted in the study.

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DEDICATION

To my parents who always said - *Kudenga kuna Mwari, regai nditaure ndichiti Mwari ariko kudenga (*There is God in Heaven, let me say again God is there in Heaven*).*

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LIST OF ABBREVIATIONS

ºC	Degree Celsius
%	Percentage
α	Alpha
β	Beta
µg	Microgram
µL	Microlitre
µM	Micromolar
3′	Three Prime
5′	Five Prime
ACC	Acetyl-CoA carboxylase
AD	Alzheimer's disease
ADA	American Diabetes Association
ATP III	Adult Treatment Panel III
ACAT	CoA: cholesterol Acyltransferase
AGE	Advanced glycation end product
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BS	Bile salts
BMI	Body Mass Index
Bp	Base Pairs
C	Cytosine
CCF	Congestive Cardiac Failure
CCK	Cholecystokinin
CDC	Centre for Disease Control
CE	Cholesterol esters
CETP	cholesteryl ester transfer protein
CHBAH	Chris Hani Baragwanath Academic Hospital
CHD	Coronary Heart Disease
CI	Confidence Interval
cm^2	Cubic Centimetre
CRP	C-reactive protein

CVD	Cardiovascular Disease
DCCT	Diabetes Control and Complications Trial
DDL	Diabetic Dyslipidaemia
DM	Diabetes mellitus (Diabetes)
DNA	Deoxyribonucleic acid
dysβ	dysβlipoproteinaemia
EDTA	Ethylenediaminetetraacetic Acid
eNOS	endothelial Nitric Oxide Synthase
EP	Evaluation protocol (EP-5 etc)
ER	Endoplasmic Reticulum
et al	et alia (and others)
F	Forward
FCH	Familial combined hyperlipidemia
FBDB-100	Familial Binding Defective Apolipoprotein B-100
FED	Fish-eye disease
FDA	Food Drug Administration
g	Gram
G	Guanine
GDM	Gestational Diabetes Mellitus
GOF	Gain-of-function
HDL	High-density lipoprotein cholesterol
HNF	Hepatocyte nuclear factor
HIV	Human Immunodeficiency Virus
HSL	Hormone sensitive lipase
HSPG	Heparan sulfate proteoglycans
HWE	Hardy Weinberg Equilibrium
IDFACE	International Diabetes Federation and American College of Endocrinology
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
Kg	Kilogram
L	Litre
LADA	Latent autoimmune diabetes of the adult

LCAT	Lecithin cholesterol Acyltransferase
LDL	Low-density lipoprotein cholesterol
LDLR	Low Density Lipoprotein Receptor
LOF	Loss-of-function
Lp(a)	Lipoprotein a
LpL	Lipoprotein Lipase
LRP	Low-density lipoprotein receptor related protein
LXR	liver X receptors
m^2	Cubic Metre
mm^2	Cubic Millimetre
mmol/L	millimoles per litre
M	Molar
mg	Milligram
MetSyn	Metabolic syndrome
MIDD	maternally inherited diabetes and deafness
MODY	maturity-onset diabetes of the young
NCDs	non-communicable diseases
NCEP	National Cholesterol Education Program.
ng	Nanogram
NGSP	National Glycohemoglobin Standardization Program
NHLS	National Health Laboratory Services
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NO	nitric oxide
NODAT	new-onset diabetes after transplantation
Non-HDL	Non-High-density lipoprotein cholesterol
nm	Nanometre
NS	Nephrotic syndrome
NPC1L1	Niemann-Pick C1-Like 1
OGTT	Oral Glucose Tolerance Test
P-value	Probability value
PDH	pyruvate dehydrogenase
PCR	Polymerase Chain Reaction
PCSK9	Proprotein convertase subtilisinlike/kexin type 9

POCT	Point of care testing
pH	Power of Hydrogen
PL	Phospholipids
PVD	Peripheral vascular disease
R	Reverse
RCF	Relative Centrifugal Force
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
RPM	Revolutions per minute
SD	Standard deviation
SGLT	sodium-glucose linked transporter
sn	stereospecific numbering
SNP	Single nucleotide polymorphism
T	Thymine
Ta	Annealing Temperature
Taq	Thermus aquaticus
TG / TAG	Triglyceride / Triacylglycerol
Tm	Melting Temperature
T1DM	Type one Diabetes mellitus
T2DM	Type two Diabetes mellitus
T3DM	Type three Diabetes mellitus
u	Units
U	Uracil
UC	unesterified cholesterol/ free cholesterol
UCT	University of Cape Town
UV	Ultra Violet
V	Volts
v/v	Volume per Volume
VLDL	Very Low-density lipoprotein
w/v	Weight per Volume
WHO	World Health Organisation
Wits	University of the Witwatersrand

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CHAPTER 1: INTRODUCTION

1.1 Background

Diabetic dyslipidemia is the abnormal lipoprotein profile found in patients with diabetes. Therefore, the presence of diabetes is a prerequisite for this definition to be valid. Patients found with dyslipidaemia have often been labelled as being secondary to diabetes; and not much work has been done to explore the possibility of underlying genetic influences. From the metabolic pathway perspective dyslipidaemia would manifest because of some malfunction in one or more of the proteins (enzymes) in the pathway. Therefore, the sequence of events expected is; i) development of diabetes, ii) reduced function of the enzymes involved in lipoprotein metabolism resulting in dyslipidaemic phenotype. The focus will be to explore the dyslipidaemia that develops in diabetes and making diabetes the first event but not the primary focus.

1.2 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases that results in high blood glucose (hyperglycemia) due to defects in insulin secretion, insulin action, or both (American Diabetes Association., 2009). The word mellitus was incorporated to distinguish DM from diabetes insipidus, a condition associated with frequent urination (Himsworth, 1936). Diabetes mellitus (DM) refers to diabetes that has no distinction between Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM). It is defined as a chronic metabolic disorder characterised by persistent hyperglycaemia. DM has become a major epidemic of this century (Shaw et al., 2010), following an increase in incidence by 50% over the past 10 years (Danaei et al., 2011). However, it is surprising given that DM is one of the world's oldest diseases, traced as far back as in ancient Egypt, Persia, and India (Ahmed, 2002: Eknoyan and Nagy, 2005). Poor glycaemic control in DM results in development of several long-term complications such as (i) dyslipidaemia, which can further complicate the cardiovascular system, (ii) peripheral vascular disease, iii) diabetic nephropathy, and (iv) ocular damage, (Kitabchi et al., 2009). Through an oxidative process, DM enhances the formation of altered LDL particles, which are atherogenic and associated with the increased risk of atherosclerosis compared to healthy individuals (American Diabetes Association, 2004; Litwak et al., 1998). DM falls into two broad categories, T1DM caused by an absolute deficiency of insulin secretion and T2DM caused by a combination of resistance to insulin

action and an inadequate insulin secretion. There are several subtypes under T2DM which include, maturity onset diabetes of the young, gestational diabetes, mitochondrial diabetes, new onset diabetes after transplant and type 3 diabetes mellitus.

1.2.1 Types of Diabetes mellitus

1.2.1.1 Type 1 Diabetes mellitus (T1DM)

T1DM results from the pancreas' failure to produce enough insulin which is caused by autoimmune destruction of β-pancreatic cells. The pathogenesis of T1DM involves several factors that include β-cell autoantigens, macrophages, dendritic cells, B-lymphocytes, and T-lymphocytes, resulting in the loss of the insulin producing β-cells of the islets of Langerhans in the pancreas and insulin deficiency (Yoon and Jun, 2005). The inheritance/familial aspect is attributed to multiple genes, including certain HLA genotypes, known to influence the risk of developing DM. In genetically predisposed individuals the onset can be triggered by one or more environmental factors, such as viral infection with some evidence showing some association between T1DM and Coxsackie B4 virus. The majority of T1DM is of the immune-mediated nature, in which T-cell-mediated autoimmune attack leads to the loss of β-cells and eventual depletion (Cnop et al., 2005). T1DM causes approximately 10% of DM cases in North America and Europe. However, no data is available on its prevalence in South Africa and Africa in general. The assumption that there is deficiency and not total absence of insulin is that sensitivity and responsiveness to insulin are usually normal, especially in the early stages. T1DM can affect both children and adults, but because the majority cases were in children it was traditionally termed "juvenile DM". T1DM can have dramatic and recurrent swings in glucose levels, making it difficult to manage; hence it was referred to as Brittle unstable DM or labile DM (Vantyghem and Press, 2006). Brittle DM has episodes of hypoglycaemia or hyperglycaemia that prevents an individual from leading a planned life style.

The swing between the opposite glycaemic levels seem to point towards several causes that can rapidly deplete or block the use of glucose. Possible causes in the swing include; i) metabolic errors resulting in an imbalance between elevated insulin releases relative to available carbohydrate, ii) possible interference from other medical conditions, iii) possible high temperature inactivation of insulin, and iv) production of insulin auto antibodies, which

at high levels can cause episodic hyperglycaemia secondary to neutralising the insulin (Schade and Burge, 1995). This gives a picture of clinical insulin resistance, a feature that can be corrected by changing the type of insulin administered (Davidson et al., 1991). Another fascinating feature of T1DM is partial remission. During this period, clinically, newly diagnosed T1DM experience a transient partial remission period ("honeymoon"), shortly after insulin treatment is initiated. During this period the patient's need for exogenous insulin decreases and in other cases, the need totally disappears with metabolic control reaching optimal levels. The mechanism or pathogenesis of this phenomenon is still puzzling, but the current thinking revolves around, i) possible combination of partial β-cell recovery with improved insulin secretion that reflects residual β-cell function and ii) an improvement of peripheral insulin sensitivity. It is interestingly to note that the residual β-cell function was reported to be highest in the age group 10–15 years (Hramiak et al., 1993).

1.2.1.2 Latent Autoimmune Diabetes of the Adult (LADA)

This is considered as a late onset subtype of T1DM, in which patients present with a clinical diagnosis of T2DM. They possess islet autoantibodies, mostly autoantibodies to glutamate decarboxylase (GAD). Patients in this category progress more rapidly to insulin treatment. This is known as latent autoimmune diabetes of the adult (LADA) and occurs in individuals with a clinical phenotype resembling T2DM but rapidly progress to T1DM requiring insulin (Irvine et al., 1976). Prevalence of LADA is not known in South Africa but studies in Nigeria (Padeola et al., 2015) reported similar clinical and lipid profiles to patients in Ghana (Agyei-Frempong et al., 2008) and among Caucasian populations.

1.2.1.2.1 T1DM and dyslipidaemia

Dyslipidaemia is a major contributing risk factor for cardiovascular disease in adult T1DM patients (Berenson et al., 1998). The pathophysiology of dyslipidaemia in T1DM is frequently a function of insulin deficiency and poor glycaemic control as well as peripheral hyperinsulinisation caused by intensive control. The process that leads to cardiovascular complications begin early at a youthful age (Lawson et al., 1999). HbA1c and waist-to-height ratio (WHtR) also called waist-to-stature ratio (WSR) are modifiable risk factors associated with change in dyslipidaemia over time in youth with T1DM (Shah et al., 2017). WHtR, defined as waist circumference divided by height, both measured in the same units gives an

index of the distribution of body fat.

1.2.1.3 Type 2 Diabetes mellitus (T2DM)

T2DM begins with insulin resistance, a condition in which cells fail to respond properly to insulin and therefore, as the disease progress, a lack of insulin may also develop (Kasuga, 2006). T2DM mellitus accounts for 80–90% of all DM cases. It is a heterogeneous disorder in which 5–10% of the patients may have maturity-onset DM of youth (Fajans, 1989), 5–10% may have latent adult-onset autoimmune DM and another 5–10% may have DM secondary to rare genetic disorders (Moller and Flier, 1991). The cause for the remaining 70–85% typical T2DM remains obscure. However geographical differences in the incidence T2DM suggest that it is a heterogeneous pathology with strong genetic and environmental contributions (Kahn, 1994). The presence of several abnormal genes or polymorphisms for the development of the disease is consistent with polygenic inheritance. Secretion and utilisation/sensitivity of insulin, which are both genetically controlled, play a crucial role in the pathogenesis of T2DM (Iselius et al., 1985). Most diabetic patients have central obesity, which is associated with insulin resistance/insensitivity (Bouchard, 1995). Such complexity requires distinction between i) diabetogenic genes, involved in the development of DM and ii) DM related genes which ii) regulate appetite, energy expenditure, and intraabdominal fat accumulation.

Lifestyle factors associated with T2DM and obesity include lack of physical activity, poor diet, stress, and urbanisation (Straznicky et al., 2011). Rural to urban migration in South Africa and Africa in general brings with it several challenges such as jobs, accommodation, transportation, water and sanitation that can induce severe stress. In 2014 the MRC of South Africa reported that the highest rate of obesity and overweight among adults in sub-Saharan Africa was found in South African women at 42%, while the combined rate of both overweight and obesity is 69.3%. South African men showed a 39% overall prevalence rate, with only a 14% obesity prevalence (Keating et al., 2014).

1.2.1.3.1 Maturity Onset Diabetes of the Young (MODY)

MODY is very rare compared with T1DM and T2DM and can therefore be mistakenly diagnosed. The most common types of MODY are hepatocyte nuclear factor1-alpha (HNF1-alpha), a gene that causes about 70 per cent of cases of MODY; HNF4-alpha which is not as common as the other forms of MODY. Children with this type of MODY may have a

birth weight around 4 kg. HNF1-beta is the type of MODY associated with several complications that include renal cysts, uterine abnormalities and gout. Glucokinase, the enzyme associated with glucose level monitoring is an example of an enzyme coded for by a diabetogenic gene that causes alteration in the activity of glucokinase (Steele et al., 2013). The common mutation results in reduced insulin secretion but may not be sufficient to cause DM in most individuals unless there is an increased demand for insulin. A mutation results in higher than normal glucose levels (Horikawa et al., 1997). MODY can be suspected and recognised if a T2DM–like condition occurs in two to three or more generations and the pattern of inheritance is consistent with autosomal-dominant inheritance (Fagans, 1990). Dyslipidaemia in MODY has been reported to be lower compared to T2DM with types like HNF1-alpha having lipid constituents similar to non-diabetics (Schober et al., 2009; McDonald et al., 2012).

1.2.1.3.2 Gestational diabetes mellitus (GDM)

GDM is diagnosed in the second or third trimester of pregnancy in an individual who did not have DM prior to gestation (American Diabetes Association, 2007). GDM is a common metabolic disease during pregnancy affecting approximately 7% (range: 2–18%) of all pregnancies (Buckley et al., 2012). Features such as both inadequate insulin secretion and insulin sensitivity resemble T2DM. Some patients return to normal glucose levels after delivery, while 5–10% of those affected remained diabetic, mostly with T2DM (Kim et al., 2002). GDM is fully treatable but requires careful medical supervision throughout the pregnancy. If untreated, GDM can be a risk to optimal development of the fetus and might result in DM related complications to the mother (Vambergue and Fajardy, 2011). Risks to the baby include macrosomia (high birth weight), congenital heart and central nervous system abnormalities, and skeletal muscle malformations. Increased levels of insulin in a fetus' blood may inhibit fetal surfactant production and cause respiratory distress syndrome (Allen and Palumbo, 1981). A high blood bilirubin level may result from red blood cell destruction. Prevention and treatment involve a healthy diet, physical exercise, not using tobacco and being a normal body weight. Blood pressure control and proper foot care are also important for people with the disease. Type 2 DM may be treated with oral medications, which may be augmented with insulin (Petznick, 2011). Weight loss surgery has been reported to be an effective measure in obese T2 DM patients (Buchwald et al., 2009). The number of people with DM is expected to rise to 592 million by 2035 (Guariguata et al., 2014).

DM in non-pregnant women is associated dyslipidaemia, consisting of low high-density lipoprotein (HDL), increased triglycerides, and postprandial lipemia (Ginsberg, 1996). Normal pregnancy is associated with changes in lipid metabolism, increasing lipid levels as gestation progresses. Both TC and TG rise throughout pregnancy with TG rising disproportionately in comparison to other lipid fractions reaching two to four times pre-pregnancy levels by the third trimester (Hadden and McLaughlin, 2009). Therefore, when coexisting as seen in GDM, dyslipidaemia would be expected to be a prominent feature.

1.2.1.3.3 Mitochondrial diabetes (MIDD)

Around 1% of all cases of DM are due to mutations in the mitochondrial DNA (mtDNA) known as mitochondrial diabetes. This is a heterogeneous syndrome which results from an A to G substitution at the conserved position 3243 (*m.3243A>G*) of the mitochondrial DNA. This is often associated with deafness and is collectively, known as maternally inherited diabetes and deafness (MIDD) syndrome (Martinez et al., 2017). Patients with MIDD are often misclassified as T2DM or T1DM because physicians are unaware of the syndrome. The presence of DM, deafness and a family history of the same clinical features in maternal relatives raise the suspicion of MIDD. The age and the mode of presentation of DM results in misdiagnosis as either T1DM or T2DM. Some cases have an insidious onset similar to T2DM, but approximately 20% of cases present acutely, with a small proportion presenting with ketoacidosis (Murphy et al., 2008). MIDD patients lack the autoimmunity, pancreatic auto-antibodies and high-risk HLA polymorphisms associated with T1DM (Kavvoura, 2014).

Mitochondrial dysfunction has been reported to contribute towards insulin resistance in both classic and non-classic insulin target tissues. In addition to insulin resistance, mitochondrial dysfunction is also associated with metabolic and cardiovascular abnormalities resulting in cardiovascular disease. Furthermore, interventions that improve mitochondrial function also improve insulin resistance (Befroy et al., 2007).

1.2.1.3.4 New-Onset Diabetes after Transplantation (NODAT)

The stress of surgery and post-surgical use of immune-suppressive medications can result in

hyperglycaemia in the recipients. NODAT a complication following organ transplant has been reported to occur in between 2% and 53% of transplanted patients (Pham et al., 2007). Hyperglycaemic state may not be persistent in all cases and therefore, referred to transient NODAT for the state of hyperglycaemia that started and ended within the first year of transplantation (Cosio et al., 2005). The incidence of NODAT is reported to be 18.0% in renal transplant in South Africa (Alagbe et al., 2017) and 27.4% of liver transplant in Egypt (Zayed et al., 2017). There may be several factors that contribute to the development of NODAT, but the use of immunosuppressive calcineurin-inhibitors like tacrolimus and cyclosporine both with diabetogenic effects are associated with an incidence of NODAT (Panz et al., 2002). The mechanism involves enhanced glucose absorption in the jejunum by upregulated expression of sodium-glucose linked transporter (SGLT1) induced by tacrolimus as well as induced insulin resistance (Li et al., 2015). The major complication in post-transplant is morbidity and mortality attributable to CVD and NODAT an independent predictor of cardiovascular events (Yates et al., 2012).

1.2.1.4 Type 3 diabetes mellitus (T3DM)

T3DM is also known as brain DM because the brain requires insulin to form new memories. Receptors on the brain's synapses facilitate the communication that creates new memories and insulin produced by the brain prevents amyloid β-derived diffusible ligands that destroy the receptors (Steen et al., 2005). In T3DM the brain either does not produce enough insulin for new memory formation or is resistant to the insulin it produces. This leads to inability to form new memories resulting in T3DM with symptoms, signs and difficulties that mimic those of Alzheimer's and dementia. The similarity of these diseases makes it difficult to diagnose T3DM unless specifically using Magnetic Resonance Imaging (MRI) scanning technology. T2DM and impaired fasting glucose has been noted to occur more frequently in patients with AD than normal population. Dyslipidaemia reflecting disordered lipid metabolism with a high degree of unsaturated fatty acids when compared to traditional Alzheimer determined by genetic predisposition has been reported in the brain of patients with T3DM (Ginneken et al., 2017). ApoE is a cholesterol carrier in the brain, helping in amyloid aggregation and the clearing of deposits from the parenchyma of the brain. When its function is reduced, excessive beta-amyloid deposits occur in the brain (Huang et al., 2004).

1.2.3 Diagnosis of DM

The Kidney has different threshold levels for components in the blood and therefore when the glucose concentration in the blood reaches its threshold it will be excreted in the urine, a phenomenon referred to as glycosuria. As an osmotically active substance the osmotic pressure of the urine increases, thus inhibits reabsorption of water causing increased urine production commonly referred to as polyuria. Loss of extracellular fluid (water) causes blood volume reduction and the intracellular fluid moves to extracellular space. The loss of fluid from the cells results in reduction in cell sizes (dehydration). All the four major stimuli to thirst, hypertonicity through the osmoreceptor, hypovolaemia, through low pressure baroreceptors, hypotension through high pressure baroreceptors and angiotensin II trigger the thirst centre resulting in the need to drink water commonly referred to as polydipsia. Following an extensive exercise by the American Diabetes Association's (ADA), World Health Organisation (WHO) published a revised classification and diagnostic criteria for DM shown in Table 1 below (Diabetes Care, 1997). According to this criterion, fasting glucose levels from 6.1 to 6.9 millimoles per litre (mmol/l) (110 to 125 milligram/deciliter (mg/dl) falls under impaired fasting glucose (American Diabetes Association, 2005), plasma glucose at or above 7.8 mmol/l (140 mg/dl), but not over 11.1 mmol/l (200 mg/dl), two hours after a 75-g oral glucose load falls under impaired glucose tolerance. Of these two prediabetic states, the latter is a major risk factor for progression to DM, as well as cardiovascular disease. Fasting is a preparation for sample collection the following day to assess the basal metabolic state of the organ. However, this does not give a real-life daily scenario and glycated haemoglobin offers a more real time monitor of carbohydrate metabolic events over a long time and this, therefore, is better than fasting glucose (Goldstein et al., 2003). In South Africa, the diagnosis and management of DM (SEMDSA, 2012) is very closely aligned to the (World Health Organisation) WHO protocol in Table 1.1 below and the American Association of Diabetes (ADA) (Amod et al., 2012). Therefore, in this project reference is based on the WHO and ADA studies.

Table 1.1 WHO revised diagnostic criteria for DM (American Diabetes Association, 1997)

WHO diagnostic criteria				
Condition	2-hour glucose	Fasting glucose	HbA_{1c}	
Unit	mmol/l(mg/dl)	mmol/l(mg/dl)	IFCC mmol/mol	NGSP/DCCT %
Normal	<7.8 (<140)	<6.1 (<110)	<42	<6.0
Impaired fasting glycaemia	<7.8 (<140)	≥6.1(≥110) & <7.0(<126)	42-46	6.0–6.4
Impaired glucose tolerance	≥7.8 (≥140)	<7.0 (<126)	42-46	6.0–6.4
Diabetes mellitus	≥11.1 (≥200)	≥7.0 (≥126)	≥48	≥6.5

Note: HbA1c – Glycated haemoglobin; DCCT – Diabetes Control and Complications Trial; IFCC – International Federation of Clinical Chemistry; mmol/l – millimoles/litre; mg/dl –milligram/deciliter; NGSP – National Glycohemoglobin Standardisation Program.

1.2.3.1 Prediabetes

Prediabetes also referred to as intermediate hyperglycaemia is defined based on glycaemic parameters above normal but below DM thresholds. This is often associated with T2DM and the term prediabetes has been criticised because it implies that DM is imminent but many of them do not develop and it also gives an impression that the absence of disease does not require any intervention. Impaired fasting glucose (IFG) is the stage in which the blood sugar level during fasting is repeatedly higher than the accepted normal levels, but the levels fall below DM levels (Nichols et al., 2007). There is 50% risk over 10 years of progressing to overt type 2 DM for IFG if there are no modifications to the lifestyle. Prediabetes was defined in 1997 by the American Diabetes Association to facilitate classification of such individuals (American Diabetes Association, 2005). Impaired Fasting Glucose (IFG) diagnosis is made before commencing the Oral Glucose Tolerance Test (OGTT), but Impaired Glucose Tolerance (IGT) diagnosis can only be made when the OGTT is done. In IGT, subjects have blood glucose levels that are higher than normal but not high enough to warrant a diagnosis of DM. Thus, oral glucose tolerance test (OGTT) is central for its diagnosis. Two-hour glucose levels of 7.8 to 11.0 mmol/l (140 to 199 mg/dL) on the 75-g oral glucose tolerance test confirm a diagnosis of IGT (American Diabetes Association, 2005). Both IFG, IGT and other metabolic derangements occur during the transition to DM.

It may take several years before the development of DM, but what is known is that up to 70% of subjects with such pre-diabetic states eventually develop DM (Knowler et al., 2002; Larson et al., 2004). A meta-analysis involving over 1.5 million prediabetes individuals reported an increased risk of cardiovascular related pathologies like coronary heart disease, stroke, as well as all-cause mortality in the subjects (Huang et al., 2016).

1.2.4. Glycated Haemoglobin

Glycated haemoglobin also referred to as haemoglobin A1c, HbA1c, A1C, or Hb1c was initially identified as an "unusual" haemoglobin in patients with DM nearly 50 years ago (Rahbar et al., 1969). After that discovery, numerous small studies were conducted correlating it to glucose levels with a conclusion that HbA1c could be used as an objective measure of glycaemic control, was introduced for clinical use in the 1980s, and subsequently became critical in clinical practice (Massi-Benedetti, 2006). HbA1c formed in a non-enzymatic pathway when haemoglobin is exposed to high plasma levels of glucose (Koenig et al., 1976). The glycation products are formed both inside and outside the cells. Glucose attaches to the amino groups of the proteins through the non-enzymatic process to form schiff base and Amadori products (Schmidt et al., 1994; Singh et al., 2001). Haemoglobin is one such protein glycated in such a reaction. The glycated haemoglobin builds up within the red cell therefore reflecting the average level of glucose to which the cell has been exposed during its life cycle of 120 days. The HbA1c level is proportional to average blood glucose concentration over the previous three months (Nathan et al., 2007). The three months' period is based on the lifespan of red blood cells (RBCs) which is four months (120 days), but not all red blood cells will undergo lysis at the same time. Once haemoglobin molecule is glycated, it remains in that state and therefore a build-up of glycated haemoglobin within the red cell reflects the average level of glucose to which the cell has been exposed during its life cycle. Glycation must be distinguished from glycosylation, an enzymatic process that results in the linkage of sugars to certain amino acid residues such as serine and asparagine. Enzymatic glycosylation is important for several functions, such as differentiating proteins, increasing protein half-life during circulation and formation of specific receptors for different ligands. Therefore, glycation a non-enzymatic process must be differentiated from glycosylation an enzymatic process (Brownlee, 1995).

The clinical significance of HbA1c test was confirmed in patients with DM (Genuth, 1995). The HbA1c reference range for patients with good glucose control is ≤6.5% according to the

International Diabetes Federation and American College of Endocrinology (IDFACE) and ≤7.0% for the American Diabetes Association (ADA). Both the DCCT and the UKPDS demonstrated that the HbA1c could predict the risk of microvascular complications in patients with DM. These two large studies demonstrated that reduction of HbA1c was associated with a significantly slower progression of microvascular disease (DCCT, 1993; UKPDS, 1998) The DCCT study reported that 10% reduction in HbA1c levels resulted in a 43%–45% lowering of risk of retinopathy. HbA1c values given in Diabetes Complications and Control Trial percentage (DCCT %) units can be correlated to the average glucose in plasma circulation as an estimated average glucose (eAG) using the equation: eAG (mg/dl) = 28.7 × A1C − 46.7 or eAG (mmol/l) = 1.59 × A1C − 2.59 (Nathan et al., 2008). On average, HbA1c of 6% corresponds to mean plasma glucose of 7.6mmol/L (135 mg/dl). For every increase in HbA1C of 1%, mean plasma glucose increases by 1.9mmol/L (35 mg/dl). Non-diabetic subjects have an HbA1C between 3.5% and 5.5%. The glycation rate is 50% during the first 30-60 days prior to the HbA1C measurements and another 25% and during the 60-120 days (Beach, 1979).

The presence of several different haemoglobin-glucose adducts like A1a (HbA1a), HbA1b, and HbA1c compounded by the presence of different variants resulted in the development of several different analytical methods. These factors lead variation in reference intervals and results reported by different laboratories. The publication of the DCCT revealed that the lack of standardisation of HbA1c methods produced very wide variability among methods, with values ranging from 4.0% to 8.1% on the same blood sample (Little et al., 1992). Using a standardisation process based on the DCCT reference method, the NGSP promoted a dramatic improvement in comparability of HbA1c values among laboratories. This resulted in the adoption of NGSP-certified methods by many laboratories (Goldstein et al., 2004)

However, some clinical conditions may result in either falsely high or low HbA1c levels. Patients with poorly controlled DM are expected to have high HbA1c, but such patients may have low or normal HbA1c if there is an underlying condition that shortens the life span of the red cell causing increased red cell turnover (Nitin, 2010). The increased red cell turnover shortens the exposure of the cell to glucose, resulting in lower HbA1c levels. Such conditions include acute and chronic blood loss, hemolytic anemia, and splenomegaly. In these situations, it may be preferable to use an alternative measure of glycemic control, such as

fructosamine or glycated albumin (Freedman et al., 2010). On the other hand, conditions that prolong the life span of the erythrocyte expose the cell to glucose for a longer period resulting in higher HbA1c levels. These conditions include vitamin B-12 and folate deficiency anemias, and asplenia (Kilpatrick et al., 1998). Iron deficiency anaemia has also been associated with higher HbA1c levels, which decrease once treatment for the anaemia has commenced (Brooks et al., 1980). Racial and ethnic differences in HbA1c and blood glucose have also been described but the reasons for these differences are still speculative (Herman and Cohen, 2012). Due to these factors, some researchers are sceptical about using HbA1c on its own for the diagnosis of DM and therefore, encourage the inclusion of traditional glucose criteria when screening for and diagnosing DM.

1.2.4.1 Use of Point of Care Testing (POCT) for HbA1c

HbA1c point-of-care instruments have greatly improved availability of results for patient management. This has gone a long way in preventing treating "history" when patients return a few weeks later for management review based on results of the tests. However, a faster result is not necessarily equivalent to traditional, core laboratory testing. Preanalytical, analytical and post-analytical factors can influence the quality of POCT that could lead to misinterpretation. The assays employed are generally less analytically sensitive than assays performed in the central laboratory (Nichols, 2003). For POCT analyses, achieving an imprecision of 2% CV is difficult to meet and therefore, and imprecision of 3% CV would be acceptable even though not optimal (Shephard, 2006). An evaluation of eight HbA1c POCT instruments was conducted according to CLSI protocols EP-10 for imprecision, EP-5 for accuracy and EP-9 for bias was conducted to assess compliance to NGSP criteria. The exercise showed that only two, the DCA Vantage and other analyzers met the acceptance criteria of having a total CV 3% in the clinically relevant range (Holmes et al., 2008). At CHBAH and surrounding clinics, Chemical Pathology POCT team of Medical laboratory technicians, manages POCT.

1.2.5. The Prevalence of DM

As of 2015 there were approximately 392 million people diagnosed with the T2DM compared to around 30 million in 1985 (Smyth and Heron, 2006). Urban drift and adoption of Western lifestyles, has predisposed African people to risk factors associated with non-communicable diseases (NCDs). As a result, NCDs like DM are rising rapidly in Africa

(Aspray et al., 2000; Murray et al., 1997). DM previously considered rare in Africa is now reaching epidemic levels with some epidemiologists predicting that the economic impact of DM, as well as the consequent death toll, will soon surpass HIV and AIDS (Chijioke et al., 2010; Kasiam et al., 2009). The developing world is experiencing the biggest increase of DM, and by 2025, more than half of people with DM will be found in developing countries (King et al., 1998). In 2000, the prevalence of DM in the World Health Organisation (WHO) African Region was estimated at 7.02 million people, including 0.702 million (10%) people with T1DM and 6.318 million (90%) with T2DM. CDC National Diabetes Statistics Report pointed that 29.1 million or 9.3% of the population in United States have DM. Of these 21 million had confirmed diagnosis but 8.1 million people 27.8% were undiagnosed (CDC, 2014).

The rise in DM will eventually lead to a rise in DM related complications like dyslipidaemia and stroke will increase compared to non-diabetic subjects. Of serious concern is the link between DM and tuberculosis. Individuals with DM have an increased risk the risk of developing tuberculosis and individuals with tuberculosis-DM co-morbidity are now more than those with tuberculosis-HIV co-infection (Ronacher et al., 2015). Previously, there was no concern because there was an apparent separation in the affected communities, the developed (western countries) affected by DM and developing countries (Africa, India and South America) affected by tuberculosis. The epidemic rise of DM in the developing counties is now of serious concern because it will negatively impact on the control and treatment of TB.

According to statistics, published on 30 October 2012, there are about 52 million people in South Africa and 51% were females. The diversity of the population is shown by the fact that 79.6% of the population is black, 9% coloured, 9% white and 2.5% Indian/Asian. With a population of nearly 13.2 million people, Gauteng province is home to nearly a quarter of the population (Statistics South Africa, 2012). According to a South African burden of disease study the overall prevalence of DM is estimated to be about 5.5% in people older than 30 years of age, (Pi-Sunyer et al., 2007).

1.2.6 Pharmacological Management of Diabetes

In South Africa, the pharmacological management of DM is outlined in the 2012 Society for

Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) Guidelines for the Management of T2DM (Amod et al., 2012). The protocol is very closely aligned to the 2016 ADA revised protocol (ADA, 2016). The generic baseline management under both the SEMDSA and ADA are changes in lifestyle that include health eating, physical activity with an aim to control weight and DM education.

The first step, metformin a biguanide marketed under the tradename Glucophage is the recommended drug. Metformin exerts its effect by activating adenosine monophosphate (AMP) kinase, resulting in the reduction of hepatic glucose production (Amira et al., 1990). Additional effects include improved peripheral glucose utilisation, reductions in gastrointestinal glucose absorption and enhanced incretin responses. Step two is dual therapy, if HbA1c is greater than 7 % after three months or more on metformin a second drug like a sulphonylurea, thiazolidinediones, dipeptidyl peptidase 4 [DPP-4] inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists) or Sodium glucose cotransporter 2 (SGLT2) inhibitors is added. In our clinic and the public-sector health institutions in general, sulphonylureas drugs are available with very few if any offering thiazolidinediones as the second drug. The other drugs GLP-1 receptor agonists and DPP4 inhibitors are not available as the second drug.

The other option available under this category is that the patient is maintained on oral medication but basal insulin, either intermediate or long-acting is given at night between 21h00-22h00. The sulphonylurea mechanisms of action involve inducing insulin release by binding to specific receptors on the pancreatic β-cell-KATP channel. The β-cell-KATP channel is a hetero-octamer, comprising a potassium channel (Kir6.2) and a sulphonylurea receptor (SUR1). The binding of sulphonylureas to SUR1 leads to glucose-independent closure of the potassium channel, membrane depolarisation, the opening of calcium channels, and the release of stored insulin (Proks et al., 2002). Thiazolidinediones (TZDs) also known as glitazones (peroxisome proliferator-activated receptors (PPARs)) are a new class of drugs for the treatment of T2DM. They are nuclear receptors, which avidly bind to peroxisome proliferator-activated receptors gamma (PPARγ) in the adipose tissue stimulating adipogenesis and fatty acid uptake. When activated, they bind to DNA in complex including retinoid X receptor (RXR), another nuclear receptor resulting in the increase of transcription of a number of specific genes and decreasing transcription of others. They enhance storage of

fatty acids in adipocytes, thus decreasing dependence on fatty acids for energy and increasing utilisation of carbohydrates, mainly glucose for energy (Jerry and Donald, 2004). Dipeptidyl peptidase-4 (DPP4) inhibitors, prevent the degradation of endogenous GLP-1 by DPP4. GLP-1 exerts its main effect by stimulating glucose-dependent insulin release from the pancreatic islets. It increases incretin levels which in turn inhibit glucagon release resulting in increased insulin secretion. Endogenous GLP-1 has a short half-life of one to two minutes, as a result of rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4) and therefore GLP-1 levels can be raised therapeutically using intravenous GLP-1 agonists that are resistant to enzymatic degradation, or by oral DPP-4 inhibitors. GLP-1 levels are abnormally low in patients with T2DM (Doyle and Egan, 2007; Thornberry et al., 2009). Sodium glucose cotransporter 2 (SGLT2) inhibitors are the newest class of oral agents for the treatment of T2DM. SGLT inhibitors block the SGLT2 protein involved in 90% of glucose reabsorption in the proximal renal tubule leading to increased renal glucose excretion and thus reducing blood glucose levels (Nair et al., 2010).

Step three is triple therapy, if HbA1c is greater than 7 % after three months or more on dual therapy, the patient is commenced on a combination of any of the three drugs in step two.
Step four is a combination of the injectable (insulin) in the form of basal insulin at night and meal time insulin if HbA1c is greater than 7 % after three months or more on triple therapy.

1.3 General lipid and lipoprotein metabolism

In order to appreciate how dyslipidaemia occurs in DM, and how DM affects proteins/enzymes involved in lipoprotein metabolism, general lipoprotein metabolism will be discussed. The lipid content in lipoproteins is dynamic, with the protein content being exchanged during circulation. The exchange of protein components results in one transformation from type of lipoprotein to another type of lipoprotein (Grow and Melvin, 1978). The composition and ratio of constituents of the lipoproteins determines their chemical, physical and electrical properties. High triglyceride content results in larger but less dense lipoproteins while high TC content results in smaller but denser lipoproteins (Kostner and Alaupovic, 1972). Lipid and lipoprotein metabolism involve processes of exchange of protein components between lipoproteins resulting in formation of new lipoproteins. The process includes i) transfer of apolipoproteins between lipoproteins, ii) transfer of lipids between lipoproteins, iii) metabolism of lipid content by enzymes, iv) hydrolysis of TG by

lipoprotein lipase (LpL), and esterification of UCl by lecithin cholesterol acyl-transferase (LCAT) and v) final clearance of lipoproteins from the circulation by the liver through their receptors. All these processes can be categorised into three pathways exogenous, endogenous and reverse pathways; and dyslipidaemia develops when any of the components in these pathways are compromised. The pathways are intricate with some overlapping aspects. Therefore, some enzymes/proteins and lipoproteins that feature prominently in one of the three pathways will be briefly discussed in one of the pathways including roles played in diabetic dyslipidaemia.

1.3.1 Exogenous Pathway

In view of the importance of postprandial lipaemia, this pathway will be discussed in detail. The pathway is important in handling the dietary acquired lipids, becoming active in post absorptive until one to five hours after a meal. Dietary fat is mainly composed of TG. Lipases hydrolyse lipids; lipase secreted by the tongue and soft palate and gastric mucosa can hydrolyse up to 30% of fats in the stomach (Hill, 2012). These may have a significant role when there is pancreatic dysfunction as they do not require bile salts (BS) and are active at gastric pH (Hill, 2012). Dietary lipids in the small intestines stimulates the release of CCK (Cholecystokinin), an enzyme which stimulates gall bladder contraction, resulting in the release of bile salts and the simultaneous release of pancreatic digestive enzymes. Secretin in the upper duodenum but found throughout the ileum, stimulates the release of a bicarbonate rich fluid that neutralises the acidic contents from the stomach (Hill, 2012). CE is hydrolysed by cholesterol esterase while phospholipids are hydrolysed by phospholipase A2.

With the aid of bile salts the digestive products form micelles that convey the non-polar lipid molecules from the lumen of the gut to the epithelial cell surface where absorption is facilitated by fatty acid binding protein. In the enterocyte, fatty acids are incorporated into TG, which, together with phospholipids, TC and CE and specific apolipoproteins (ApoB-48 and ApoA-1) are assembled into spherical chylomicrons which are released by exocytosis into the intestinal lacteals (Holmes and Lobley, 1989). Apo B48 a specific marker of intestinal chylomicron particles has amino acid sequence which represents 48% of the initial sequence of ApoB-100. It is synthesised by the intestinal mucosal cells and is the permanent structural protein of a chylomicron (Adiels et al., 2012). The lacteals drain into the cisterna chyli and thence to the thoracic duct. Chylomicrons finally enter the subclavian vein for systemic circulation. Absorption of dietary fatty acids is usually complete whereas absorption

of total gut cholesterol (biliary and dietary) is variable. Once in the systemic circulation, chylomicrons donate A-I to HDL in exchange of ApoCII and ApoE. When ApoCII is present on the surface of chylomicrons, activates lipoprotein lipase situated on the capillary endothelial surfaces. LpL hydrolyses TG to yield monoacylglycerol and free fatty acids (Carey et al., 1992).

The fatty acids released into circulation associate with albumin to return to the liver if not taken up, can be taken up by organs such as muscle (energy source) or adipocytes (storage). During chylomicron circulation which can last from 5 to 30 minutes, they rapidly deliver TG to peripheral tissues. No chylomicrons should be present in the plasma of a fasting subject six to eight hours after a meal. The metabolism of TG rapidly depletes the core of the chylomicron particle, reducing its size and increasing its density. In the circulation, cholesteryl ester transfer protein (CETP) shuttles between HDL and apoB-containing lipoproteins and facilitates the bi-directional transfer of CEs and TG between them and these changes result in loss of affinity for ApoC lipoprotein which partitions to HDL (Kontush et al., 2015). The chylomicron remnant contains less TG and much more CE than the original chylomicrons, making it highly atherogenic. Only ApoB 48 and ApoE remain on the chylomicron remnants and the latter serves as ligand for the hepatic receptors (Cooper et al., 1997). The chylomicron remnants are internalised through the LDL and LRP receptors by endocytosis. The components of the chylomicrons are hydrolysed in the lysosomes and the cholesterol released from the hepatic lysosomes can enter pathways for the formation of bile acids, be secreted into the bile as such, be incorporated in nascent lipoproteins or esterified with long fatty acids and can down-regulate HMG CoA-reductase, the rate limiting enzyme of cholesterol biosynthesis. Postprandial hypertriglyceridemia is a pro-atherogenic marker for predisposition to atherosclerosis and macrovascular disease in T2DM subjects (Kumar et al., 2010). Measurement of ApoB48 as a marker of chylomicron metabolism can be used to assess the integrity of this pathway (Nakajima et al., 2014). Overcoming the TG components in this pathway and attaining the baseline metabolic status was the rationale for the patients to fast when assessing lipids and lipoproteins profiles. Assessment of cholesterol does not require fasting because there is a stringent enterocyte cholesterol homeostasis involving rates of sterol synthesis, efflux, and uptake from gut lumen. On the apical surface of enterocytes there is active influx of unsterilized cholesterol (UC) as well as unesterified phytosterols into the enterocyte through the Niemann-Pick C1-Like 1 (NPC1L1) transporters (Jia et al., 2011).

NPC1L1 is also found in the canaliculi membrane of hepatocytes. It functions as a sterol transporter to mediate intestinal cholesterol absorption and counter balances hepatobiliary cholesterol excretion. Also, on the apical surface adenosine triphosphate (ATP)-binding cassette (ABC) transporters ABCG5 and ABCG8 complex promotes active efflux of unsterilized sterols from enterocytes back into the intestinal lumen for excretion (Jakulj et al., 2010). The enterocyte has sterol-excess sensor, the liver X receptors (LXR) that monitor sterol levels and activate the genes that regulate NPC1L1, ABCG5 and ABCG G8 (Lehmann et al., 1997). Only free or unesterified cholesterol (UC) can be absorbed through gut enterocytes. CE which constitutes more than 50% of the dietary cholesterol cannot be absorbed due to the bulky acyl side chains. To overcome this limitation, CE can be de-esterified by pancreatic lipases and esterolases, enzymes that cleave off the side branches to CE back to UC so that some dietary CE can be converted to UC for absorption. Similarly, the liver is only able to efflux UC via bile but not CE, from hepatocytes to the biliary system. The liver CE is de-esterified to UC by cholesterol esterolases to efflux into bile and ultimately the gut for excretion. Another important way for the reduction of cholesterol is its conversion to bile acid and efflux the bile through ABCB11 transports (Meier, 1995). This mechanism, in the form of bile acid sequestrants have been in use for a long time in the treatment of hypercholesterolemia. It interrupts enterohepatic recirculation of bile acids, thereby stimulating feedback mechanisms on the conversion of cholesterol into bile acids in the liver leading to lower cholesterol concentrations in the circulation. The bile acid sequestrants are known to improve glycaemic control in patients with T2DM (Sonne et al., 2014). Further understanding of the exogenous pathway provided another opportunity for the development of a second lipid lowering agent ezetimibe at the gut level that inhibit intestinal absorption of dietary and biliary cholesterol (Ahmed and Byrne, 2010).

1.3.1.1 Exogenous pathway and ApoE and or PCSK9

The ApoE that remains on the chylomicron remnants has been well established as the ligand for the hepatic receptors for their uptake by the liver (Cooper et al., 1997). PCSK9 is abundantly expressed in the intestine. In addition to the known effects on LDL, PCSK9 has been demonstrated to modulate cholesterol transport, metabolism and production of apo B-containing lipoproteins in intestinal cells (Levy et al., 2013).

1.3.2 Endogenous Pathway

The endogenous pathway involves metabolism of i) intestinally derived lipoproteins as well as ii) de novo derived lipoproteins. Plasma clearance of intestinally derived remnant lipoprotein by the liver involves sequestration and internalisation (Mahley and Hussein, 1991). Chylomicron and VLDL remnants pass through the fenestrae of hepatic sinusoidal endothelial cells into the space of Disse. The fenestration acts as a dynamic bio-filter that restricts the entry of large chylomicrons while allowing the smaller remnants to enter (Cooper, 1997). The initial clearance by the liver begins with sequestration of the remnants within the space of Disse, where ApoE secreted by hepatocytes enhances remnant binding and uptake. Heparan sulfate proteoglycans (HSPG) abundant in the space of Disse mediate the enhanced binding to facilitate the action of LpL on the remnants also present in the space of Disse (Hamilton et al., 1990; Stow et al., 1985). Molecules taken up by the liver bind to proteoglycans as well as lipoprotein receptors such as LRP for internalisation or uptake. For the intestinally derived remnant lipoprotein, the uptake by hepatocytes includes, i) LDL receptors mediated direct uptake (Ishibashi et al., 1996), ii) the HSPG-LRP pathway mediates uptake either by transfer of the remnant from the HSPG to the LRP for internalisation or by binding of the remnant lipoproteins to HSPG forming a tertiary complex with the LRP that is then internalised. The HSPG are critical in the HSPG-LRP pathway because if absent, the remnants do not bind and iii) the HSPG alone can mediate remnant lipoprotein uptake (Mahley and Ji, 1999). The LDL receptor remnant uptake is independent from both the HSPG and the LRP. After internalisation, remnants are catabolised in the lysosomes with the various remnant catabolic products entering various metabolic pathways.

In its early stages, the endogenous pathway resembles the exogenous pathway but instead of chylomicrons, the liver produces VLDL in a process that involves a combination of TG and cholesteryl esters and apolipoprotein B-100, resulting in their transportation to the peripheral tissues (Shelness and Sellers, 2001). During circulation, the nascent VLDL particles interact with HDL particles resulting in HDL particles donating apolipoprotein C-II and apolipoprotein E to the nascent VLDL. Similar to chylomicrons the addition of apolipoproteins C-II and E to nascent VLDL results in its maturity. The Apo C-II on the VLDL surface activates endothelial LpL (Goldberg, 1990), which hydrolyses TGs into FFAs and glycerol that are taken up by cells. Just as with chylomicrons, the VLDL-C is transferred

to HDL, after esterification. Gradually all of the apo C-II and some of the apo E are returned to HDL. After the last of the apo C-II has returned to HDL, the remaining particles are known as IDL. Intermediate-density lipoproteins (IDL) are the product of LpL processing of VLDL and chylomicrons. IDL are smaller than VLDL. They contain equal amounts of TG and TC (30% TG, 30% TC) and a moderate amount of protein (15%). The permanent structural protein is apo B-100, and IDL also still contain apo E. More than half of the IDL is taken up by the LDL receptor, which recognises apo B-100 and apo E. The rest is converted to LDL by the action of hepatic TG lipase (HTGL), located in the vascular endothelium of the liver, which removes some of the TG. After the last of the Apo E has returned to HDL, the remaining particles are known as LDL that is even smaller than IDL. LDL contains predominantly cholesterol (50%) and protein (25%) and a very small amount of TG (5%). In healthy individuals six LDL subfractions with mean density ranging from 1.0268 to 1.0597 g/ml can be identified. The major differences in chemical composition of the subfractions are in the cholesteryl ester to triglyceride ratio which gives them different metabolic and atherogenic roles (Shen et al., 1981). The permanent structural protein is apo B-100, which is the only protein in LDL that has a long half-life of 3 days. LDL delivers cholesterol to peripheral tissues and are eventually taken up by the LDL receptor (at low LDL levels) or by the macrophage scavenger receptor (at high LDL levels).

In de novo endogenous pathway, cholesterol is synthesized from acetyl Co-A in the liver regulated by HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase. In negative feedback response the enzyme is inhibited by increased intracellular cholesterol derived from the degradation of internalised LDL. LDL is internalised via the LDL receptor and therefore, competitive inhibition of the enzyme will result in abrogation of the negative feedback response and induce the expression of LDL receptors in the liver. The increased LDLR will in turn increase the catabolism of plasma LDL and lowers the plasma concentration of cholesterol. This mechanism is the rationale behind the actions of statins. The reductase gene transcription is regulated by the sterol regulatory element binding protein (SREBP). When activated, SREBP binds to the sterol regulatory element (SRE) found on the 5' end of the reductase gene, but when SREBP is inactive, it binds to the ER or nuclear membrane with another protein called SREBP cleavage-activating protein (SCAP). When cholesterol levels are low, SREBP is released and migrates to the nucleus, where it binds to the SRE and

transcription is enhanced. When intracellular cholesterol increases, proteolytic cleavage of SREBP from the membrane ceases and any proteins in the nucleus are quickly degraded.

1.3.2.1 Endogenous pathway and ApoE and or PCSK9

ApoE has been associated with increased cholesterol during normal pregnancy (Tanyanyiwa et al., 2016) and in the general population it was associated with increased cholesterol and LDL (Masemola et al., 2007). The endogenous pathway is affected by PCSK9 through the LDL clearance process. Both gain and loss of function PCSK9 mutation have an impact on the type of dyslipidaemia that develops. Gain of function mutations reduces the number LDLR through inhibition of endocytic recycling of LDLR back to the plasma membrane (Zhang et al., 2007). In addition to its role as receptor ligand molecule for chylomicrons in the pathway (Cooper, 1997), ApoE modulates the LpL-mediated processing of triglyceride-rich lipoproteins in the endogenous pathway. LpL-mediated hydrolysis is attributed to the positive arginine residues present on ApoE (Rensen and van-Berkel, 1996).

1.3.3 Reverse Pathway

This deals with the transport of cholesterol in the cells from the peripheral tissues to the liver. Nanscent/Newly formed HDL is synthesised in both enterocytes in the liver and from the chylomicron surface membrane liberated during chylomicron metabolism (Brunham et al., 2006). Nascent HDL is a phospholipid disc with no lipid core but during its circulation, accept UC from peripheral tissues and circulating VLDL and LDL resulting in a small spherical HDL3-C particle. Movement of UC from cells is mediated by ATP-binding cassette transporter A1 (ABCA1) which then combines with apoprotein A-I (apo A-I), the structural protein for HDL to produce nascent HDL (Oram, 2002). LCAT, which esterifies the surface UC provides neutral lipid that forms the core. When the surface cholesterol is esterified, it enters the nonpolar core of HDL thus, leaving the surface of HDL free to acquire more cholesterol from lipoproteins and cell membranes. The size of HDL particles increases as cholesterol esters accumulate (HDL2). The liver, through a putative HDL receptor delivering cholesterol, clears some of the HDL2. HDL then transports cholesterol to where it is needed, mostly via CETP, and the liver (for clearance). Some of the HDL2 is cleared by the liver via an HDL receptor and most of HDL2 is cycled back to HDL3, by transferring the CE back to the TG-rich chylomicron remnants in exchange for TG via CETP. Therefore, the overall

effect of HDL is anti-atherogenic because excess cholesterol in living and dead cells is returned to the liver by this reverse cholesterol transport pathway.

1.3.3.1 Reverse pathway and ApoE and or PCSK9

HDL is involved in both ApoE and PCSK9 through the presence of ApoE on HDL molecules. ApoE in lipoproteins acts as a ligand of LDLR family proteins and promotes lipoprotein particle clearance. ApoE is an efficient cholesterol acceptor in HDL, and the binding of ApoE in newly secreted HDL increases the particle size (Gordon et al., 1983). PCSK9 mediates regulation of ApoE levels in HDL concentration (Choi et al., 2013). The high PCSK9 and CETP activity in patients with metabolic syndrome is directly proportional to the increasing number of components that constitute metabolic syndrome. The positive correlation between PCSK9 levels and the CETP activity could be that higher PCSK9 concentrations inhibit LDLR recycling resulting in higher LDL plasma. As a substrate, the higher LDL levels triggers increased CETP activity. Conversely, higher CETP activity would elicit higher LDL and PCSK9 concentrations (Girona et al., 2016). CETP activity is significantly increased in familial hypercholesterolemia patients with PCSK9 Leu181Arg gain-of-function mutation (Abifadel et al., 2012).

1.4 Apolipoprotein E

Apolipoprotein E (ApoE) is a 299-amino acid peptide with a molecular weight of 34kilo Daltons. The 3.7-kilo bases in length ApoE gene is found on chromosome 19 and contains 4 exons. ApoE is normally present in serum at approximately 5-8 mg per deciliter (Lusis et al., 1987). Approximately three quarters of the plasma ApoE is synthesized in the liver, in the hepatic parenchymal cells where it is incorporated into VLDL. Other organs, including the brain, lungs, spleen, adrenals, ovaries, kidneys, muscle cells and macrophages also produce small amounts of ApoE (Fazio et al., 1992). The structure of ApoE is divided into three, an amino terminal end made up of 165 residues is highly ordered, and the next 35 residues make up a random structure and the carboxyl terminal, which is also highly ordered again. The Carboxyl terminal forms the strongest lipid binding area. The structure is made up of approximately 62% alpha helices, which are amphipathic and important in lipid binding. While also providing a more hydrophilic aspect to the water environment, the rest of the secondary structure is made up of beta sheets (9%), beta turns (11%) and random structure (18%) (Mahley, 1988). The five arginine and three lysine residues between 140 and 160 are

essential for binding to the LDL receptor. ApoE is the primary ligand for several lipoprotein receptors, and therefore, plays a crucial role in the clearance of lipoproteins from the circulation. Chylomicron and VLDL remnants, which are rich in CE than the original chylomicrons contain ApoE that serves as ligand for the hepatic receptors. The chylomicron remnants are internalised through the LDL and LRP receptors by endocytosis (Havel et al., 1998). Its lipid clearance role makes it an important determinant of plasma cholesterol and potential biomarker for cardiovascular disease risk (Bennet et al., 2007). Studies have shown that mice lacking ApoE (ApoE-/-) accumulate cholesterol-rich remnant particles with plasma cholesterol reaching very high levels and accelerated development of atherosclerosis (Pendse et al., 2009).

1.4.1 Apolipoprotein E isoforms

ApoE was first isolated from plasma in 1973 and was originally known as arginine-rich apolipoprotein. There are three common isoforms of ApoE designated E2, E3 and E4 according to their charges in isoelectric focusing. The main differences between the isoforms shown in Table 1.2 below are at the amino acid residues 112 and 158. ApoE2 has cysteine at both residues and it has the lowest affinity for the LDLR, with less than 2% of normal receptor binding activity. Apo E3 has a cysteine at residue 112 and arginine at 158 and has a much better receptor binding activity. ApoE4 has arginine at both these residues and shows 100% normal receptor binding activity and hence, it has a very rapid clearance from HDL, VLDL and Chylomicrons (Mahley, 1988). ApoE3 and ApoE4 both have normal LDLR building activity as the R at 158 forms a salt bridge with aspartate at 154 leaving the series of positively charged R and K between 130 and 148 often to interact with the negatively charged as on the LDLR.

Table 1.2 ApoE: Amino acid residue positions and their charge

Isoform	E2/2	E3/3	E4/4
Relative Charge	0	+1	+2
Amino Acid on 112	Cysteine	Cysteine	Arginine
Amino Acid on 158	Cysteine	Arginine	Arginine

1.4.1.1 Minor ApoE isoforms

Minor ApoE isoforms have been identified: ApoE1 was found to have similar lipid profile to ApoE2 and ApoE5 was located as a band in the basic region relative to apoE-4 on an isoelectric focusing gel (Ordovas et al., 1987). ApoE7 also known as ApoE-Suita found predominantly in the Japanese population is associated with hyperlipidemia and atherosclerosis (Yamamura et al., 1999).

1.4.2 ApoE Nomenclature

Electrophoretic analysis of ApoE showed that it is composed of numerous isoprotein components (Utermann et al., 1982). The complex structure of ApoE stems from both genetic polymorphism and post-translational sialylation modifications. Genetic studies have shown the existence of alleles at two loci that specify six different ApoE phenotypes, three homozygous and three heterozygous (Zannis and Breslow, 1981). Early isoelectric focusing technique resulted in a nomenclature system (Utermann et al., 1977), which was different from later analysis on two-dimensional polyacrylamide gel electrophoresis (Zannis and Breslow, 1981). The difference between the isoelectric and polyacrylamide findings gave rise to the creation of a uniform system that describes the ApoE isoproteins, alleles, genotypes and phenotypes. The ApoE alleles are ε4, ε3 and ε2 while the major asialo ApoE isoproteins seen in plasma by two-dimensional gel electrophoresis is designated ApoE4, ApoE3 and ApoE2 respectively. ApoE4 is the most basic while ApoE2 is the most acidic ipoprotein. The minor plasma ApoE isoproteins that can be eliminated by treatment with neuraminidase are collectively designated ApoEs. Thus, the sialo ApoE isoproteins of ApoE4, ApoE3 and AapoE2 are designated ApoE4s, ApoE3s and ApoE2s respectively. Distinction between sialo apoE isoproteins can be achieved by adding a number after the subscript as ApoE3s1, ApoE3s2 and ApoE3s3 (Zannis and Breslow, 1981).

There are six well characterised ApoE genotypes comprising three homozygous states (*E-4/4*; E-3/3 and E-2/2) and three heterozygous (*E-4/3*; *E-3/2* and *E-4/2*) states. ApoE and ε allelic frequency shows a variation among populations around the world as shown in Table 1.3. Apo-3/3 phenotype is the most common (ranging 50-70%) and the ε3 allele makes up a large majority of the ApoE gene pool (typically 70-80%) of the population. The high frequency of ε3-allele lead to the belief that it was the wild type, but the presence of ε4 allele in nearly all animal species makes it the most likely ancestral allele.

Table 1.3 Ethnic distribution of ApoE					
Population and Location	**ApoE2**	**ApoE3**	**ApoE4**	**N**	**Study**
Caucasian (Western Europe)	10	75	15	2020	Lucotte et al., 1997
Zimbabweans (Southern Africa)	16	62	23	690	Tanyanyiwa, 2005
Pygmies (Central African Republic)	6	54	41	70	Zekraoui et al., 1997
Khoi San (Bushmen) South Africa	8	55	37	247	Sandholzer et al., 1995
West, Central and East Africans.	12	71	18	470	Zekraoui et al., 1997

Several studies on the ApoE gene reported varying allele frequencies across the world between 1995 and 2007. As reflected in Table 1.3 above ApoE3 is the most frequent allele (Hallman et al., 2007). Interestingly, in studies from Europe there is a north to south decline in the e4 allele, with a higher frequency in the northern region and a lower frequency in the southern region (Gerdes at al., 1996). The frequency of the e3 allele is lower in African population than the Caucasians, but e4 allele is higher than in Caucasian populations. The high frequency of ApoE3 allele seems to imply that it is a product of mutational changes of e4 allele, which is found in high frequencies the African ancestral tribes (Corbo and Sacchi, 1999). The high frequency of e4 allele among the population groups where the food supply was scarce could be an evolutional protective adaption in scarce food situation as it increases absorption of cholesterol and plasma cholesterol levels. Therefore, it is not surprising to find the e4 allele in high frequencies in the oldest populations known as hunter-gatherers like the Khoi San and African Pygmies. Several studies looked at ApoE in relation to variable pathologies, but no record of studies looked at its behaviour or effects in DM.

1.4.3 Binding Properties

The binding properties and mechanism show how ApoE is associated with dyslipidaemic states. The heparin-binding domain between 131 and 150 amino acids of ApoE is responsible for the high affinity binding to the LDL and LRP receptors (Ji et al., 1997). Receptor binding

is due to the ionic interaction between basic amino acid residues in this region and acidic amino acid residues of the LDL receptor. The LDL receptor possesses seven repeated segments that include critical acidic amino acids aspartate and glutamate near the N-terminus representing the ligand-binding site. The LRP contains 31 domains, homologous to the ligand binding sites of LDL receptor (Brown and Goldstein, 1983). Both residues 112 and 158 are outside the binding region of ApoE. The binding affinity cannot be directly linked to residue 112, but residue 158 can have an influence on the binding affinity depending on the salt bridge it forms with other amino acids. The basic amino acids in the 131 – 150 regions are largely solvent exposed extending away from the backbone of the helix, forming a basic field of charge that may be available to interact with the receptor (Beisiegel et al., 1989). The backbone structures of ApoE2 and ApoE3 are essentially identical. However, there are local changes in the region of the residue 158. In ApoE3, there is a salt bridge between arginine 158 and aspartate 154. ApoE2 has a neutral amino acid cysteine rather than the arginine residue at 158 thus, prevents the salt bridge formation resulting in aspartate interacting with arginine 150, forming a new salt bridge (Weisgraber et al., 1982).

This interaction swings the side chain of arginine 150 into a new plane outside the receptor-binding region and disrupts receptor binding because arginine is part of the receptor-binding region. Therefore, the substitution at residue 158 of Apo E2 appears to have a secondary effect on the receptor-binding domain of ApoE, affecting binding indirectly (Lalazar et al., 1988). All autosomal dominant ApoE mutations cause defective LDL receptor binding, but their LDL receptor binding activities are higher than those of autosomal recessive ApoE2 mutations. ApoE2 Arg145Cys mutation was reported to produce dysβ in the predominantly black African subjects of Xhosa descent (de Villiers et al., 1997). If present, these subjects would fall in the mixed dyslipidaemia phenotype category. Another interesting aspect in the African population is the ApoE-2/2 genetic status associated with dysβ, which reduces the rate of remnants clearance. Under normal physiological conditions, subjects with E2/2 have lower plasma cholesterol; and dyslipidaemia related to reduction in remnant clearance is not apparent. The mechanism involved is that the production rate of remnants and their clearance rates are balanced because the normal levels of VLDL results in low levels of VLDL remnants. The E2/2 low receptor affinity leads to low influx of cholesterol rich VLDL remnants resulting in LDLR up regulation in response to lower influx of remnants into the liver. The upregulated LDLR results in increased cholesterol internalisation leading

to lower plasma cholesterol compared to other ApoE isoforms. Conditions like DM that result in increased production of VLDL remnants and hypothyroidism that result in generalised reduced clearance producing the dysβ phenotype, hence the conclusion that an additional 'hit/stressor' is still required for dyslipidaemia to become evident.

1.4.3.1 ApoE and dysβ-lipoproteinaemia

Dysβ also known as broad β disease or type III hyperlipidaemia and remnant removal disease is a highly atherogenic dyslipidaemia associated with certain ApoE variants (Smelt and Beer, 2004). Premature or accelerated atherosclerosis occurs in one third to more than one half of individuals with Dysβ. Peripheral vascular disease involving the lower extremities is almost as common as coronary artery disease in Dysβ. This is different from the distribution of vascular disease seen in familial hypercholesterolemia in which there is less involvement of the lower extremities. Although the mechanism underlying the predisposition for atherosclerosis of peripheral vessels in type III is unknown, it is worth noting that certain cholesterol-fed animals with high levels of β-VLDL have a higher incidence of peripheral vascular disease than of coronary atherosclerosis (Mahley et al., 1985). The association of homozygous ApoE-2/2 with dysβ resulted in numerous investigations for the underlying mutations or variants in patients presenting the dyslipidaemic picture. Known common variants causing dysβ are shown in Table 1.4 below.

Table 1.4: Some apo E allele variants associated with dysβlipoproteinaemia

Allele Variant	Inheritance	LDL-R Binding	Researchers
158Arg→Cys	Recessive	2%	Emi et al., 1988.
136Arg→Ser	Unknown	40%	Emi et al., 1988 & Wardell et al., 1987
142Arg→Cys	Dominant	20%	Smit et al., 1990
145Arg→Cys	Dominant	45%	de Villiers et al., 1997
146Lys→Gln	Dominant	40%	Smit et al., 1990
E3(Leiden).	dominant	defective binding	Havekes et al., 1986

Key: Arg – Arginine; Cys – Cysteiene; E3 – ApoE3; LDL-R – Low density lipoprotein receptor

These variants are rare, and a lot of work is still being done especially in the eastern world (Corbo and Sacchi, 1999). ApoE1 has been associated with severe type III

hypolipoproteinaemia (Mann et al., 1995). ApoE5 has two common variants both associated with high cholesterol but with different effects on the cardiovascular disease (Wardell et al., 1991). ApoE7 is interesting because it is associated with increased plasma lipid levels and accelerated atherosclerosis due to the defective binding to LDL receptors but has preferential association with very low-density lipoproteins (VLDL), (Dong et al., 2000). ApoE an essential mediator of lipid metabolism in normolipidemic patients plays a major role in diabetic dyslipidaemia. The common dyslipidaemic phenotypes associated with both T1DM and T2DM includes increases in VLDL triglycerides, decreases in HDL, increased smaller/denser LDL, reduced clearance of postprandial chylomicrons, reduction in lipoprotein lipase (LpL) activity, and decrease in LDLR (Goldberg, 2001). It has been and still remains a problem trying to separate the lipid-independent effects of DM on cardiovascular disease from the DM induced hyperlipidemia cardiovascular complications (Wu and Huan, 2007; Johnson et al., 2010). The ApoE modulation of diabetic dyslipidaemia is through its central link between the processes of glucose and lipoprotein metabolism (Isezuo et al., 2003). ApoE4 has been shown to be less inhibitory to glycation and oxidation compared to the ApoE3 but more inhibitory than ApoE2 isoform. Modifications like glycation and oxidation to the cells of the vessel wall and LDL particles that are atherogenic occur more frequently in the diabetic setting (Shuvaev et al., 1999; Miyata and Smith, 1996; Mabile et al., 2003,). Animal experiments demonstrated that mice expressing ApoE3 were less susceptible to atherogenesis than those expressing E4 (Altenburg et al., 2007).

1.5 Proprotein Convertase Subtilisin Kexin 9 (PCSK9)

PCSK9 also known as neural apoptosis regulated convertase, NARC-1 and was initially identified as a proprotein convertase family with a role in the regeneration of the liver and differentiation of cortical neurons (Seidah et al., 2003). PCSK9 gene is located on the small arm of chromosome 1p32 and contains 12 exons and 11 introns (Seidah and Prat, 2007). PCSK9 regulates LDL receptors which are plasma membrane glycoproteins responsible for the removal of LDL particles in circulation (Brown and Goldstein, 1985). Its involvement in lipid metabolism became apparent when those with autosomal dominant mutations developed hypercholesterolemia (Abifadel et al., 2003). The development of hypercholesterolemia is due to the inability of LDLR recycling leading to high levels and prolonged circulation of LDL-C. The prolonged circulation results in increased exposure to free radicals, hence susceptibility to oxidation and risk to coronary heart disease (Abifadel et al., 2003). The normal feedback mechanism of increasing synthesis of the LDLR is effective only when

intracellular cholesterol is low (Brown and Goldstein, 1997), because cholesterol is delivered into the cell and PCSK9 acts as chaperone directing LDLR to its degradation (McNutt et al., 2007). Studies have identified separate routes involved in PCSK9 induced LDLR degradation (Poirier et al., 2007). The extensively studied mechanism of action associated with hypercholesterolemia involves PCSK9 binding to the first epidermal growth factor-like repeat (EGF-A) of LDLR. When LDL binds to the LDLR on which PCSK9 is attached, the PCSK9-LDL receptor complex is internalised. After internalisation, the PCSK9 component inhibits endocytic recycling of LDLR back to the plasma membrane resulting in lysosomal degradation of the complex (Zhang et al., 2007). The acidic environment in the endosome increases the PCSK9 affinity for the LDL receptor. The epidermal growth factor (EGF)-like repeat A of the LDL receptor region has been shown to be essential for recycling of the LDL receptor from endosomes to the cell surface when there are no molecules binding at its site (Sun et al., 2012). Its role in cholesterol metabolism made its inhibition a prime target for treating hypercholesterolemia (Park et al., 2004; Maxwell and Breslow, 2004; Benjannet et al., 2004). One of early methods used was blocking the activity of PCSK9 with an antibody. The inhibition prevents the degradation of LDLR thus, enhancing their recycling to the surface of the cell (Chan et al., 2009). This increases the clearance of LDL from the circulation. Inhibition of PCSK9 was reported in improving the dyslipidaemia seen in T2DM patients as well as non-diabetics (Sattar et al., 2016). The other PCSK9 variant is associated with increased number of LDL receptors. This enhances removal of circulating LDL leading to lower levels in plasma, a feature associated with reduced risk of coronary heart disease (Cohen et al., 2006; Zhao et al., 2006). The phenotype of hypercholesterolemia associated with reduced number of receptors and hypocholesterolaemia associated with increased number of receptors resulted in reports and investigations into PCSK9 mutations.

1.5.1 PCSK9 Mutations

PCSK9 gene mutations associated with dyslipidaemia can be classified into two major groups gain or loss of function as shown in Figure 1.1 below.

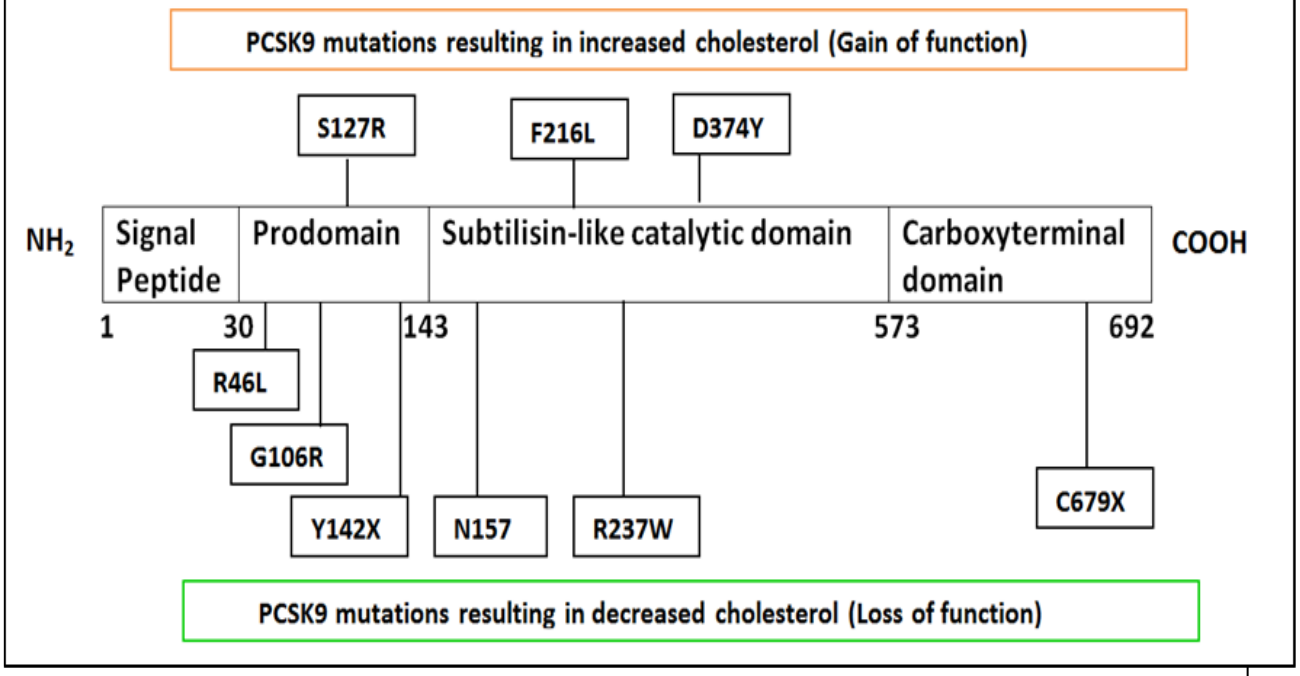


Figure 1.1 Common PCSK9 Mutations
Schematic representation of common PCSK9 mutations. Gain of function displayed at the top of the frame and loss of function mutations at the bottom of the frame.

These mutations increase LDLR levels on the surface of the hepatocyte, resulting in increased LDL clearance and therefore low plasma LDL levels. The loss-of-function mutations were identified in African-Americans with nearly 1 in 50 having one of two Y142X and C679X nonsense mutations in PCSK9, which lowers LDL levels by nearly 30% (Cohen et al., 2006; Hallman et al., 2007). A separate missense mutation that results in lowering of LDL by nearly 15% was identified in Caucasians. Besides lowering cholesterol levels there was a significant reduction in coronary heart disease (Cameron et al., 2006; Yascara et al., 2014). Even though the loss of function mutation appears to have no adverse consequences, it would be interesting to assess the effects of statin induced upregulation of PCSK9 (Dubuc et al., 2004; Rashid et al., 2005; Careskey et al., 2008) in diabetic patients especially in the Southern African population where loss of function mutation has been reported (Hooper et al., 2007). Four loss-of-function mutations have been identified and well described, showing some ethnic differences, with one of two nonsense mutations Y142X and C679X being

predominant in the African population; while the Q152H, R46L, L10A53V and I474V PCSK9 variants are found in Caucasians (Cohen et al., 2005). Both L10A53V and I474V produce dyslipidaemia in the form of low LDL levels, but they have different serum PCSK9 concentrations. Such variations point towards the presence exonic variant with different effects on LDL levels (Abifadel et al., 2009). It is also possible that there are different mechanisms of action in these two variants and therefore PCSK9 measurement might not be a marker as a measure of its function (Mayne et al., 2013). A study in Africa reported increased plasma TC and LDL levels in E670G carriers than in non-carriers and E670G increased their risk for coronary artery disease (Slimani A et al., 2014).

The second group is those causing hypercholesterolemia secondary to the enhanced function of the protein (gain-of-function). These mutations decrease LDLR levels on the surface of the hepatocyte, resulting in a reduction of LDL clearance, thus increasing their plasma levels. The identification of two gain-of-functions increased interest in PCSK9 research, and the revelation that FH was not only confined to LDLR mutations (Abifadel et al., 2003).

1.5.2 PCSK9 and DM

Until the recent Mendelian randomisation study (Schmidt et al., 2017) and PCSK9 SNP association with T2DM (Lotta et al., 2016), this project was the first to look at the possible association between T2DM and PCSK9 variants. In the Mendelian randomisation study, it was observed that four PCSK9 variants rs11583680, rs11591147, rs2479409, and rs11206510 demonstrated some associations with increased fasting glucose and WHR. The variants associated with lower LDL demonstrated an increased risk of T2DM (Schmidt et al., 2017). Emerging reports of an association between alleles influencing lipid metabolism and the risk of DM shows that those carrying alleles causing familial hypercholesterolemia have a lower incidence of DM compared to unaffected family members (Besseling et al., 2015).
A positive correlation between PCSK9 levels and HbA1c in patients with T2DM has been reported but such correlation was absent in non-diabetic patients (Yang et al., 2016). These findings are significant because non-diabetics are expected to have normal HbA1c. Another correlation study reported elevated PCSK9 levels in untreated FH patients, especially the homozygous patients. In these patients high-dose statin therapy further increased PCSK9 levels (Raal et al., 2013). PCSK9 inhibitors are effective in lowering LDL and many studies suggest a positive association of plasma PCSK9 levels with glycaemic parameters and risk of

T2DM. However, the comprehensive the impact of PCSK9 inhibitors on glycemic control T2DM remains unclear (Momtazi et al., 2017).

1.6 Assessment of possible gene-gene (ApoE-PCSK9) interactions

Studying two genes (ApoE and PCSK9) whose variants are independently associated with pathology provides an opportunity to interrogate the phenomenon of gene interactions. In gene interactions, specific alleles of one gene mask or modify (enhance, suppress or in some way alter) the expression of alleles of a second gene. If the interaction is synergistic, the contribution of two mutations to the phenotype of a double mutant exceeds the expectations from the additive effects of the individual mutations (Perez et al., 2009). Sometimes two genes produce a phenotype that might not be the expected product after taking into consideration the individual gene effects. For instance, if an ApoE gene variant associated with dyslipidaemia is found in a subject carrying a PCSK9 gene also associated with dyslipidaemia, the phenotype would be expected to have dyslipidaemia similar to a subject with a homozygous gene for dyslipidaemia. In genetic interaction, the results of two different genes can reveal the functional relationships between genes and pathways (Mani et al., 2008). In qualitative genetics the term epistatic was first used to describe a masking effect where a variant or allele at one locus prevents the variant at another locus from manifesting its effect. However, in quantitative genetics, epistasis refers to a deviation from additivity in the effect of alleles at different loci with respect to their contribution to a quantitative phenotype, for example, dyslipidaemia (Fisher, 1918). Considering that ApoE and PCSK9 are predominantly associated with the exogenous and endogenous pathways respectively, it would be interesting to see the results of different mutations in one subject. Mathematical instruments and definitions of the genetic interaction predict four possible outcomes termed Product, Additive, Log, and Min. Under certain conditions, the outcomes follow one of the predictions but can also deviate dramatically (Boone et al., 2007).

The selection of discriminant and synergic genes has been useful for medical diagnosis and prognosis. The mathematical definitions and instruments can be very complex with the pair-wise interactions I (X 1; X 2; Y) defined as I (X1; X2; Y) =I (X1, X2; Y) −I (X1; Y) −I (X2; where I is the symbol for mutual information, I (X 1; Y) is the individual effect of gene X 1 relative to phenotype Y, I (X 2; Y) is the individual effect of gene X 2 relative to Y, and I (X

1, X 2; Y) is the joint effect of X 1 and X 2 relative to Y. A positive value of I (X 1; X 2; Y) indicates synergy, while a negative value of I (X 1; X 2; Y) indicates redundancy (Xing et al., 2017). These mathematical instruments are applied in genome wide association studies (GWAS). These studies look at methods to identify associations between genetic regions (loci) and traits (including diseases). Looking at the above formula, X1 would possibly be ApoE and X2 would be PCSK and the phenotype would be dyslipidaemia.

1.7 Dyslipidaemia

1.7.1. Classification of Dyslipidaemia

Dyslipidaemia is the presence of lipid and/or lipoprotein abnormalities characterised by high serum TG and TC increased small-LDL and subnormal HDL. For clinical and therapeutic management purposes a simpler practical system of classification categorises dyslipidaemias based on increases in cholesterol only (pure or isolated hypercholesterolemia), increases in TGs only (pure or isolated hypertriglyceridemia), or increases in both TC and TGs (mixed or combined hyperlipidaemias). However, this system fails to account for specific lipoprotein abnormalities e.g. low HDL or high LDL that contribute to disease despite normal TC and TG levels. The World Health Organisation (WHO)/Fredrickson classification is more comprehensive, it considers the pattern and relationship of lipoproteins (Fredrickson,1971). Even though it is old, its adoption by WHO signifies that it is well known and widely accepted and useful in identifying a proportion of both primary and secondary hyperlipidaemias. Dyslipidaemia is considered primary if the aetiology is a genetic defect-giving rise to a product (enzyme) that cannot optimally participate in lipoprotein metabolism. This often requires pharmacological intervention with or without life style adjustments. Modern and advanced methods have resulted in the discovery of more primary genetic causes that may result in hypolipidaemia and were therefore not classified under the WHO/Fredrickson system, which looked at hyperlipidaemia. Secondary dyslipidaemia is often arising due to failure in another system that is directly linked to lipid metabolism as seen in renal pathology, hypothyroidism, DM and obesity. Some secondary dyslipidaemias may have an underlying genetic defect with reduced penetrance or may need a hit (factors) for them to present a dyslipidaemic phenotype. In cases where it may not be possible to identify the possible genetic cause, it is cost effective to exclude secondary causes before exploring possible primary causes. In some cases, it is possible to speculate on the possible

primary cause based on the time and factors associated with the appearance of the phenotype.

1.7.2 Mechanisms of Secondary Dyslipidaemia

Secondary dyslipidaemia accounts for ±40% of all hyperlipidaemias, therefore it is important and cost effective to exclude them before venturing into the genetic route. Secondary causes can precipitate or worsen a coexistent inherited hyperlipidaemia (Carr and Brunzell, 2004). Long term complications of DM include renal pathology (nephrotic syndrome and renal failure) which can, independent of DM cause dyslipidaemia (Toyama et al., 2014), with a picture similar to the one seen in DM. As a common complication of DM and as another cause of secondary dyslipidaemia, renal pathology and the mechanism of dyslipidaemia will be briefly discussed.

1.7.2.1 Nephrotic syndrome

Nephrotic syndrome (NS) is characterised by increased permeability of the glomerulus to proteins, resulting in proteinuria greater than 2.5 g/day, presence of oedema caused by loss of oncotic pressure secondary to urinary loss of proteins. The loss triggers reflex compensatory synthesis of all proteins by the liver including lipoproteins as shown in Figure 1.2 below.

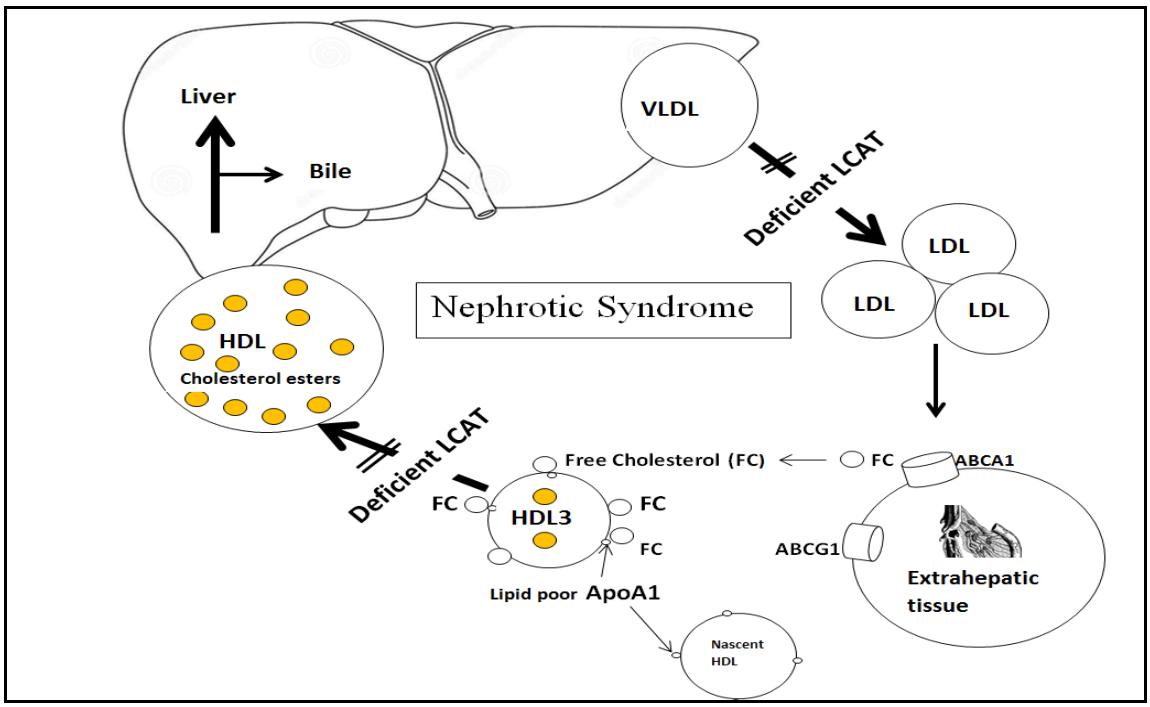


Figure: 1.2 Schematic representation of the mechanism of dyslipidaemia in nephrotic syndrome. FC – Free Cholesterol; HDL – High Density Lipoprotein; LCAT – Lecithin Cholesterol Acyl Transferase; LDL – Low Density

Non-excretion of the larger lipoproteins and loss of smaller metabolising proteins results in increased serum lipoproteins producing the dyslipidaemic picture associated with nephrotic syndrome (Attman and Alaupovic, 1990). Free cholesterol derived from cells combines with apoprotein A-I (apo A-I), a structural protein for HDL to form nascent HDL. The free cholesterol on the surface is then esterified by the actions of LCAT and internalised in HDL to form HDL3. Therefore, LCAT deficiency resulting from urinary loss in nephrotic syndrome causes accumulation of unesterified cholesterol in certain body tissues and HDL maturation (conversion of HDL3 to HDL2) is impaired and therefore, reduce/limit the cardiovascular protective, functions of HDL (Gavish et al., 1987). Hypoalbuminemia secondary to proteinuria stimulates a compensatory increased synthetic function of the liver which includes upregulation of 3-hydroxy-3-methylglutaryl CoA reductase with a consequent hypercholesterolemia. Therefore, both increased synthesis and decreased clearance of lipoproteins may contribute to the dyslipidaemia in nephrotic syndrome (Attman and Alaupovic, 1990). The urinary loss of other liporegulatory factors leads to decreased activity of lipolytic enzymes and resulting in impaired clearance of cholesterol- and triglyceride-rich lipoproteins. Increased Apo (a) biosynthesis elevates the plasma Lp (a) concentration increasing the risk for development of cardiovascular disease and thromboembolism encountered in in NS (Mahmoodi et al., 2008).

1.7.2.2 Chronic Renal Failure

There are many causes of chronic renal failure with the most common being glomerulonephritis, DM mellitus, and hypertension. However, the net effect is a progressive loss in the number of functioning nephrons. Figure 1.3 below shows the mechanism of dyslipidaemia in chronic renal failure.

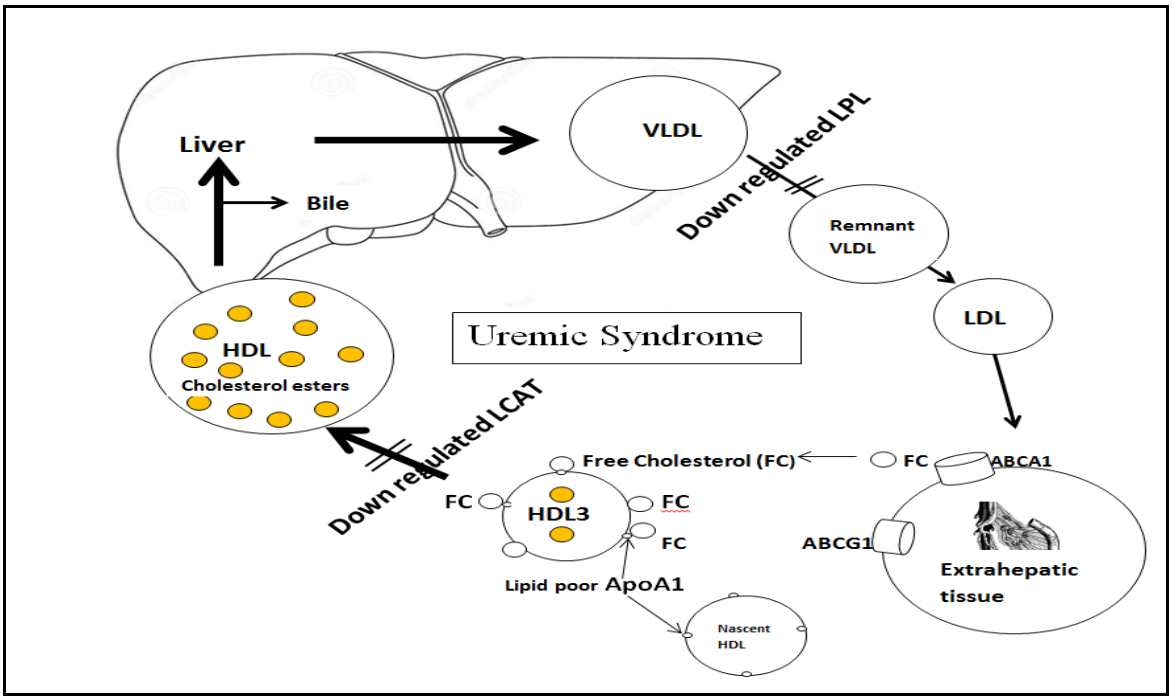


Figure 1.3 Schematic representation on the mechanism of dyslipidaemia in chronic renal failure. FC – Free Cholesterol; HDL – High Density Lipoprotein; LCAT – Lecithin Cholesterol Acyl Transferase; LDL – Low Density lipoprotein; LpL – Lipoprotein Lipase; VLDL – Very Loew Denasity Lipoprotein.

Central to dyslipidaemia in chronic renal failure is the disruption and alteration of components required for the metabolism of HDL (Vaziri, 2006). The downregulation of LCAT and upregulation of CETP result in reduction of HDL. The downregulation could be secondary to the uremic state, which is not conducive for optimal metabolic functions. The reduction in metabolic (catabolic) rate is attributed to the reduced LpL enzyme activity due to the increased presence of LpL inhibitors. Apolipoprotein C-III is a potent inhibitor of lipoprotein lipase levels increase possibly due to reduced clearance. A decreased apolipoprotein C-II, an activator LpL ratio to Apolipoprotein C-III (apolipoprotein C-II/C-III ratio) reflects the disproportionate increase of Apolipoprotein C-III (Cheung et al., 1996; Hirano et al., 2003). Therefore, the common factor in both NS and CKD is LCAT, which is deficient due to renal loss in the former and downregulated due to uraemia in the later. It has also been noted that hyperlipidemia can accelerate progression of renal disease by several mechanisms (Trevisan et al., 2006). Mesangial cells express LDL receptors and when bound LDL is oxidised, it induces mesangial cell proliferation, which in turn enhances the production of chemokines like macrophage chemo-attractant protein-1), cytokines such as

interleukin 6 and growth factors. Macrophage chemo-attractant protein-1 induces recruitment of macrophages, which can infiltrate the glomerulus and become foam cells that release cytokines. Oxidised LDL increases the adhesion of monocytes to glomerular endothelial cells, favouring monocyte infiltration, and affects tubular epithelial cells (Abrass, 2004). Hypercholesterolemia and hypertriglyceridemia are also associated with podocyte injury, which secondarily leads to mesangial sclerosis (Joles et al., 2000). Oxidised LDL induces apoptosis of podocytes and loss of a nephrin an important part of the glomerular filtration barrier. This results in increased albumin diffusion in the podocyte monolayers resulting in apoptosis (Bussolati et al., 2005). Several studies, Atherosclerosis Risk in Communities study (Munter et al., 2000), Physicians' Health Study (Schaeffner et al., 2003), the Framingham Offspring study (Fox et al., 2004) and Helsinki Heart Study (Manittari et al., 1995) show that different aspects of dyslipidaemia are associated with development of chronic kidney disease. On the other hand, some studies reported that dyslipidaemia worsened renal failure (Massy et al., 1994; Hunsicker et al., 1997). Treatment of dyslipidaemia with lipid lowering agent has been shown to improve renal function biochemistry (Athyros et al., 2003; Tonelli et al., 2003).

1.7.3 Lipids and Lipoproteins in diagnosis of dyslipidaemia

The three most commonly referred or referenced diagnostic criteria for dyslipidaemia include: i) National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATPIII); ii) World Health Organisation; and iii) European Atherosclerosis Society. In South Africa we adopted the European Guidelines for the Prevention of Cardiovascular Disease to replace the previously published South African Lipid Guidelines (Klug et al., 2012). The three systems all consider the presence of any one of the following as dyslipidaemic: TC >5.2mmol/L; LDL >3.5mmol/L; Triglyceride >1.7mmol/L; HDL <0.9mmol/L in males; and <1.0mmol/L in females. The Atherogenic index (TCl ÷ HDL) >5.0 reflects low HDL in relation to TC. Hyperlipidaemia is statistically; any lipid value above the 97.5th percentile (mean + 2 SD) and these can more specifically be classified as

i) TC: Ideal <5.2mmol/L, moderate hypercholesterolaemia 5.2 – 7.5mmol/L and severe
ii) being >7.5mmol/L and this is often seen in patients with genetic defects.
iii) TG: Ideal <1.7mmol/L, moderate hypertriglyceridaemia 2.5 – 5.0mmol/L and moderate to severe being >5mmol/L.
iv) LDL Ideal level < 3.5 mmol/L.

v) HDL Ideal level > 1.2 mmol/L.

vi) Apolipoprotein B, Ideal <100mg/dl.

vii) Apolipoprotein A, Ideal >120mg/dl

viii) Lipoprotein (a), Ideal < 30 mg/dl.

The South African National Health Laboratory Services (NHLS), the laboratory providing diagnostic services in all government health institutions incorporates an estimate of the Framingham 10-year risk of CHD in the lipid profiles reports. The Framingham risk assessment tool provides a prediction on the chances of developing cardiovascular disease in the next 10 years. The assessment tool considers the patients, age, gender, TC, HDL, tobacco smoking, presence of DM and the patient's systolic blood pressure and if the blood pressure is being pharmacologically managed (D'Agostino et al., 2008). Following the 52nd SEMDSA and 15th LASSA meeting in May 2017, treatment targets and goals for cardiovascular disease prevention for diabetic dyslipidaemia were recommended. These include TC <4.5mmoml/L, LDL <1.8mmol/L, HDL >1.0mmol/L for males and >1.2mmol/L for females, triglyceride <1.7mmol/L, systolic blood pressure <140mmHg. diastolic blood pressure <90mmHg and BMI <25kg/m^2.

Cardiovascular risk assessment science is rapidly evolving with new biomarkers, genetic tests, ratios and permutations being evaluated and being promoted for routine use. Even though these new considerations correlate significantly with cardiovascular risk, there has been no evidence to show that their inclusion to existing risk estimates, improves the predictive ability. Parallel to this study, we did a research that was accepted for publication by Central African Journal of Medicine in August 2016 reference 06/16. Analytical methods available in most public health service sector laboratories can measure TC, TGs, and HDL directly and calculate LDL from the Friedewald Equation (Friedewald et al., 1972). In this method, VLDL is estimated by TG ÷ 5 mg/dL or TG÷ 2.2 mmol/L because the cholesterol concentration in VLDL particles is usually one fifth of the total lipid in the particle. Therefore, this calculation is valid only when TGs are <4.5 mmol/l and patients are fasting, because postprandial lipids have chylomicrons which have lower VLDL. Therefore, such samples overestimate LDL. The calculated LDL value incorporates measures of all non-HDL, non-chylomicron cholesterol, including that in IDL and lipoprotein (a) Lp (a). LDL can also be measured directly using plasma ultracentrifugation (gold standard), which separates

chylomicrons and VLDL fractions from HDL and LDL Immunoassay methods are also available for the direct measurement of LDL, but these are found mainly in research, private and well-resourced laboratories. New formulas for the calculation of LDL have emerged (de Cordova et al., 2013; Hattori et al., 1998), but the Friedewald equation is still the method of choice in the region (Onyenekwu et al., 2014). Cholesterol is a negative acute phase reactant that decreases while lipoprotein (a) levels and TG increases in inflammatory states. As a negative acute phase reactant, it was previously recommended that measurement of cholesterol should be postponed until after resolution of acute illness. Lipid profiles can vary for about 30 days after an acute MI and therefore results obtained within 24 hours after MI are usually reliable enough to guide initial lipid-lowering therapy (Jackson et al., 1987).

LDL the current primary target in the management for dyslipidaemia does not seem to have reduced the progression to development of (CAD) even if the LDL levels are optimally maintained. It appears that besides the LDL constituents there are other atherogenic components. Therefore, non-HDL used in the Helsinki Heart Study3 became topical because non-HDL represents the cholesterol content present in all the atherogenic lipoproteins including LDL (Frick et al., 1987). Thus, non-HDL provides a more holistic picture of the atherogenic components.

Advantages of using non-HDL are that non-HDL incorporates all atherogenic lipoproteins, including LD, it is easily calculated from a lipid profile by a simple subtraction of HDL from TC (non-HDL = TC minus HDL) and therefore, there is no additional costs, there is no need for fasting samples as opposed to LDL, which can be affected by triglyceride in non-fasting samples (Ballantyne et al., 2001). Current management principles can be applied proportionally as high non-HDL would require increased levels of lipid-lowering agents, as well as lifestyle modification. Non-HDL management takes into account the patient's level of risk as well as the levels of LDL. Non-HDL levels are cut off and classified according to level of risk: i) ≥5.7 mmol/L is considered very high for all individuals, ii) 4.9 – 5.6 mmol/L is considered high, iii) 4.1 – 4.8 mmol/L is considered moderately high, iv) 3.4 – 4.0 mmol/L is considered near ideal, v) below 3.4 mmol/L is considered ideal for people with risk factors for coronary heart disease, and vi) ≤ 2.6 mmol/L is considered ideal for people at very high risk of heart disease and this is where the dyslipidaemic participants would be classified under. Lipid and Lipoprotein ratios seem to perform better as biological markers of

cardiovascular related dyslipidaemia than the use of the individual traditional markers (Kannel, 2005; Packard et al., 2005).

1.8 Pharmacological management of dyslipidaemia

National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults [Adult Treatment Panel (ATP)-III], approach to managing dyslipidaemia includes lifestyle changes, dietary modifications and increased physical activity. Dietary treatment of hyperlipidemia forms the baseline for the dose and frequency on which pharmacological intervention can be initiated (NCEP, 1993). DM on its own is a major cardiovascular risk factor conferring about three-fold absolute adjusted risk of CVD death (Stamler et al., 1993; Sever et al., 2005). In South Africa, the South African Heart and the Lipid and Atherosclerosis Society of Southern Africa adopted the European Society of Cardiology together with the European Atherosclerosis Society management of dyslipidaemia guidelines (Reiner et al., 2011). Based on the updated Framingham risk charts, management is based on the cardiovascular risk score and LDL levels. Patients in the very high-risk strata are not risk scored. Type 2 DM on its own and patients with chronic kidney disease with (glomeruli filtration rate (GFR) <60ml/minute/1.73m2 fall in the very high-risk group (Klug et al., 2012). The decision to treat dyslipidaemia in diabetic patients is based on the assumption that reducing the LDL from any baseline level will reduce the occurrence of cardiovascular disease (CVD). It has been clearly shown that diabetic patients should be treated with a statin with a view of maintaining LDL of 2.6mmol/L (<100 mg/dl) (ADA, 2008). In South Africa the 52nd SEMDSA and 15th LASSA meeting in May 2017 recommended treatment targets for diabetic dyslipidaemia to achieve TC <4.5mmoml/L, LDL <1.8mmol/L, HDL >1.0mmol/L for males and >1.2mmol/L for females and triglyceride <1.7mmol/L systolic blood pressure <140mmHg, diastolic blood pressure <90mmHg and BMI <25kg/m2 (Amod et al., 2017).

1.8.1 Statins

Statins have been shown to reduce cardiovascular events and all-cause mortality. Differences in structure, potency, lipophilicity and pharmacokinetics exist among the statins, but essentially, they all have the same mode of action through inhibition of the activity of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the enzyme that catalyses conversion of HMG-CoA to mevalonate, an early rate-limiting steps in cholesterol synthesis, resulting in

decreased cholesterol synthesis (Mabuchi et al., 1981). The common types of statins (brand names in brackets) with equivalent doses that can produce a 30–40% reduction in LDL respectively include: i) atorvastatin (Lipitor) 10mg, ii) Fluvastatin 80mg (Lescol, Lescol XL 80mg), iii) lovastatin (Mevacor, Altoprev 40mg), iv) pravastatin (Pravachol 40mg), v) rosuvastatin (Crestor 5mg), vi) simvastatin (Zocor 40mg), and vii) pitavastatin (Livalo 2mg) (Stender et al., 2005).

At CHBAH diabetic clinic and most public-sector health institutions simvastatin (Zocor) is the main statin used. The presence of several statins means there is variability in clinical response to treatment. The variability could be in part due to pharmacogenetics in the metabolism of statin in the pathway that is catalysed by Cytochromes P450 (CYPs) and Uridine diphosphate glucuronosyltransferases (UGT) gene family enzymes. The pharmacokinetics of individual statins depends on their hydrophobicity with the more hydrophilic statin like pravastatin require active transport into the liver and therefore not dependent on cytochrome P450 (CYP) enzymes for metabolism. Like other hydrophilic compounds the excretions is mainly renal. Less hydrophilic compounds are transported by passive diffusion and are metabolised by CYP enzymes and transported into the biliary tract for excretion (Whirl-Carrillo et al., 2012).

Besides their lipid lowering functions, statins have other beneficial properties that include improvement of endothelial function. The mechanisms in enhancing endothelial function include prevention of the downregulation of endothelial nitric oxide synthase (eNOS), an enzyme that catalyses the formation of nitric oxide (NO) from L-arginine. eNOS are inhibited by caveolin-1, which is increased by LDL and therefore the reduction of LD removes the inhibition (Feron et al., 2001). Statins have been reported to possess anti-inflammatory properties thereby reducing the risk of coronary artery disease (Ridker et al., 1998). These properties were demonstrated in the justification for the use of statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial.

1.8.2 Other lipid lowering drugs

Ezetimibe, an inhibitor of cholesterol intestinal absorption, is a lipid lowering agent commonly used in combination with a statin. It reduces low density lipoprotein (LDL) and triglyceride but increases high density lipoprotein cholesterol levels. As seem in other

combination therapies ezetimibe and statin achieves LDL targets in a greater proportion of patients than statin monotherapy. Besides the significant decrease in lipoproteins it improves glycaemic control in T2DM (Sarigianni et al., 2010). Bile-Acid Acid Binding Resins were once the major lipid-lowering drugs but are now used as adjuncts to statin therapy for patients who require further reduction of cholesterol levels beyond available statin. The commonly used drugs are cholestyramine and colestipol, whose mechanism of action is binding bile acids (not cholesterol) in the intestine. The enterohepatic circulation of bile acids is interrupted resulting in the reduction of serum LDL concentrations in patients who are already receiving a statin (Mabuchi et al., 1983).

Nicotinic acid works by inhibiting the mobilisation of free fatty acids from peripheral tissues, thereby reducing hepatic synthesis of triglycerides and secretion of VLDL. The reduction in VLDL translates to a reduction in LDL (Knopp et al., 1985). Nicotinic acid has also been given in combination with other drugs, such as a bile-acid–binding resin, fibrates and statins resulting in additional lowering of lipoproteins with a potential to reduce cardiovascular events beyond that of monotherapy (Stein et al., 1996). Fibrates, typically clofibrate (ethyl p-chlorophenoxyisobutyrate) resemble short-chain fatty acids. They increase fatty-acid oxidation in the liver resulting in increased formation of ketone bodies and decreased secretion of triglyceride-rich lipoproteins. In muscles, the increase in fatty-acid oxidation is associated with an increase in both lipoprotein lipase activity and the uptake of fatty acids. Fibrates are the most effective triglyceride lowering drugs. Fibrates also increase the buoyancy of LDL particles thus reducing levels of small dense LDL (Auwerx et al., 1996; Staels et al., 1998).

1.9 Diabetic dyslipidaemia

More cardiovascular disease occurs in patients with DM and one of the common metabolic abnormalities in these patients is dyslipidaemia with a characteristic pattern, termed diabetic dyslipidemia (Elinasri and Ahmed, 2008). Type II DM coupled with variants of proteins associated with lipid metabolism, more than one form of dyslipidaemia can be expected (Flores et al., 2003; Hansen, 2003; Gloyn, 2003; Barroso et al., 2003). A combination of the two as seen in diabetic dyslipidaemia is myriad of genetic, environmental and geographical factors involvement. With such a complex genetic picture, a complete investigation into genetics involved dyslipidaemia seen in diabetic patients would be very interesting. This was

done in this study by focusing on ApoE and PCSK9 variants. Diabetic dyslipidaemia features include reduced high-density lipoprotein (HDL), elevated triglycerides, elevated LDL and postprandial lipemia. It is interesting to note that both DM or insulin resistance and dyslipidaemia are components in the two definitions of the National Cholesterol Education Program Adult Treatment Panel III (NCEP: ATP III) and International Diabetes Federation (IDF) for metabolic syndrome (NCEP, 2001). Paralleling the unabated rise in DM is the worldwide increase in the prevalence of the metabolic syndrome. Of concern is that in Africa, there is an even higher prevalence with a rate of 43% reported for black Zimbabweans (Makuyana et al., 2004); and a rate of 60% in a clinical study of 40 urbanised South African blacks with established coronary artery disease (CAD) (Ntyintyane et al., 2006).

1.9.1 Subsets of diabetes and dyslipidaemia

The diagnosis and classification of the different subsets of DM occur in a manner that appears to be incidental and therefore, details on the specific type of dyslipidaemia that might be present has not been fully characterised. To this end, description of dyslipidaemia in DM has been confined to T1DM and T2DM. Poorly controlled T1DM is commonly associated with hypertriglyceridemia and reduced HDL (Ginsberg, 1996). The same picture is seen in T2DM except that there is reduction in size of LDL to smaller and more atherogenic species known as small dense LDL. Small dense LDL can also be seen in in prediabetes with insulin resistance but normal plasma glucose. This feature of abnormal of insulin action and not hyperglycaemia is attributed for the lipid abnormality (Haffner et al., 2000).

1.9.1.1 Lipoprotein abnormalities in DM

Defects in insulin action and hyperglycaemia can lead to disruptions in lipid metabolism resulting in a lipid phenotype associated with hyperglycaemia. T1DM provides a much clearer understanding of the relationship among DM, insulin deficiency, and lipid/lipoprotein metabolism (Hirano et al., 1991). Diabetic dyslipidaemia is a result of deficiency or absence of several factors such as insulin actions on liver apoprotein production, peripheral actions of insulin on adipose and muscle, lipoprotein lipase (LpL), actions of CETP. The production of apolipoprotein B (apoB), the major protein component of very low-density lipoprotein (VLDL) and LDL is increased in DM (Albers et al., 2008). LpL is the major enzyme responsible for conversion of lipoprotein triglyceride into free fatty acids, and hence, it is the major determinant of both serum triglyceride and HDL concentrations. In both adipose tissue

and skeletal muscle, it is insulin dependent. In insulin deficiency its activity is low but increases upon insulin therapy. In insulin deficiency, the low LpL results in elevation of total and VLDL triglyceride levels with reduction in HDL concentration (Taskinen, 1987). The release of stored fatty acids from adipocytes is a stepwise process involving conversion of stored triglyceride into fatty acids and monoglycerides that can be transferred across the plasma membrane of the cell. The rate limiting enzyme is HSL, which is inhibited by insulin. The deficiency of insulin lifts the inhibition, resulting in the characteristic features of diabetic dyslipidaemia, a high plasma triglyceride concentration, low HDL concentration and increased concentration of small dense LDL particles. These changes associated with DM mellitus are secondary to increased free fatty acid flux secondary to insulin resistance (Mooradian, 2009).

1.9.2. Mechanism of Diabetic Dyslipidaemias

1.9.2.1. Postprandial lipemia

The postprandial theory of atherosclerosis hypothesis proposed that atherosclerosis is a postprandial phenomenon that is depended on the metabolic response to the ingestion of food (Zilversmit et al., 1979). This essentially reveals the integrity of the lipid exogenous metabolic pathway, which if compromised results in increased highly atherogenic remnants. The reduced clearance of remnants results in long duration of postprandial lipaemia. The dyslipidaemia in postprandial lipaemia is characterised by increase in plasma TG due to the reduced LpL activity secondary to decreased insulin function (Kolovou et al., 2005). Subjects with type 2 DM have a slower clearance rate of chylomicrons resulting in postprandial lipemia. The interaction of the molecules involved in Figure 1.4 below, shows the main features, chylomicron remnant with ApoB48 and reduced levels of LpL. Chylomicron clearance process involves acquisition of apoCII by chylomicrons upon entry into the blood systemic circulation from the thoracic duct (Goldberg, 1990). ApoCII, a cofactor for LpL, is acquired primarily from HDL and is attached to the luminal surface of endothelium in capillaries by the protein glycosylphosphatidylinositol HDL-binding protein 1 (GPIHBP1) and by heparan sulphated proteoglycans. LpL hydrolyses the triglycerides within the chylomicron particles. Its activity is enhanced by insulin and therefore in DM the activity is reduced. Some of these patients have been found to have underlying lipid disorder, such as heterozygous LpL deficiency, that is then exacerbated by DM (Julien et al., 1997). The

formation of chylomicrons remnants is reduced by the deficiency in insulin. Therefore, there is prolonged circulation of chylomicrons resulting in prolonged post prandial lipemia. Chylomicrons contain a truncated form of apoB termed apoB48 that is 48% of full-length apoB and lacks the LDL receptor-binding site (Ebara et al., 2000).

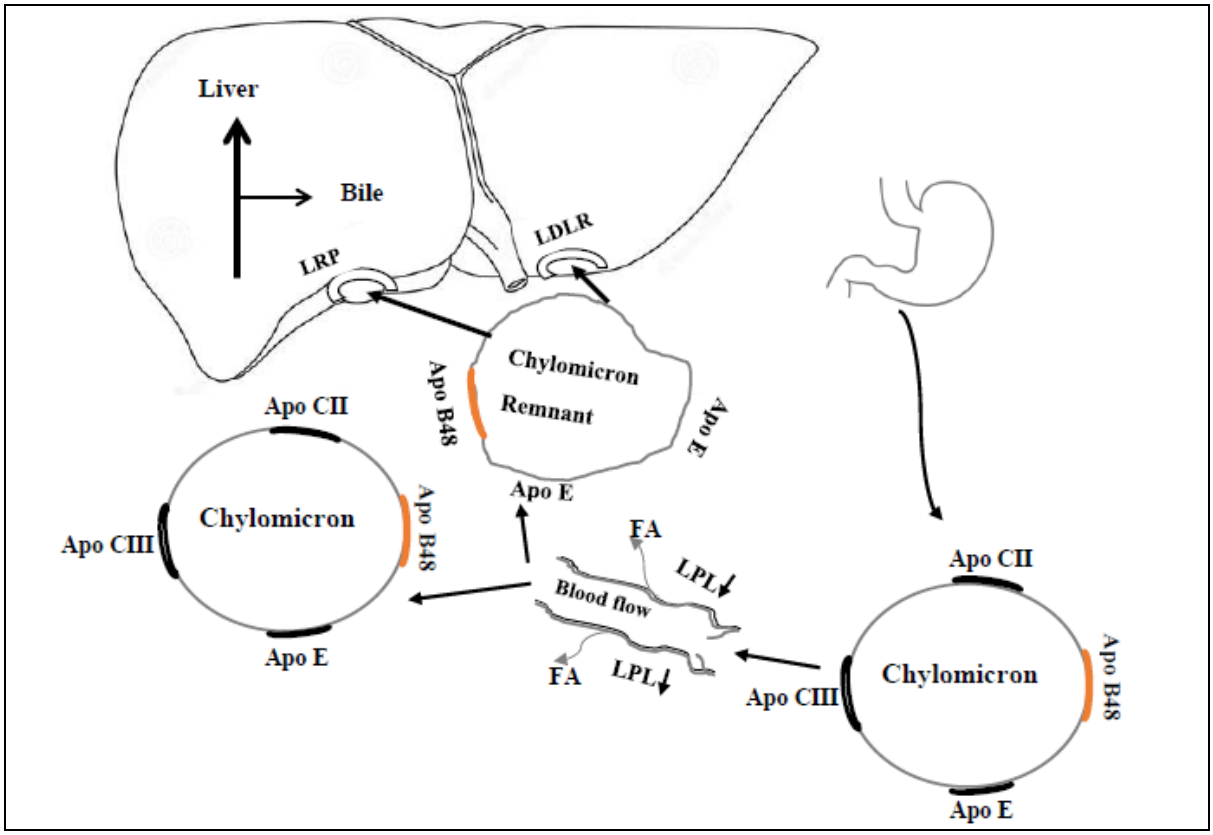


Figure 1.4: Schematic representation on the mechanism of postprandial lipaemia
FA – Fatty Acids; LDLR – Low Density Lipoprotein Receptor; ApoB – Apolipoprotein B; Apo C – Apolipoprotein C; LRP – Low Density Lipoprotein Related Peptide.

Patients with poor glycaemic control develop hypertriglyceridemia and some may develop severe hyperchylomicronaemia. At higher levels the patients can develop eruptive xanthomas and pancreatitis. Such patients require exclusion of possible underlying genetic lipid disorder, such as LpL deficiency, that may be exacerbated by DM (Julien et al., 1997).

1.9.2.2 VLDL and DM

The interaction between chylomicrons, VLDL and LpL and insulin is shown in Figure 1.5 below.

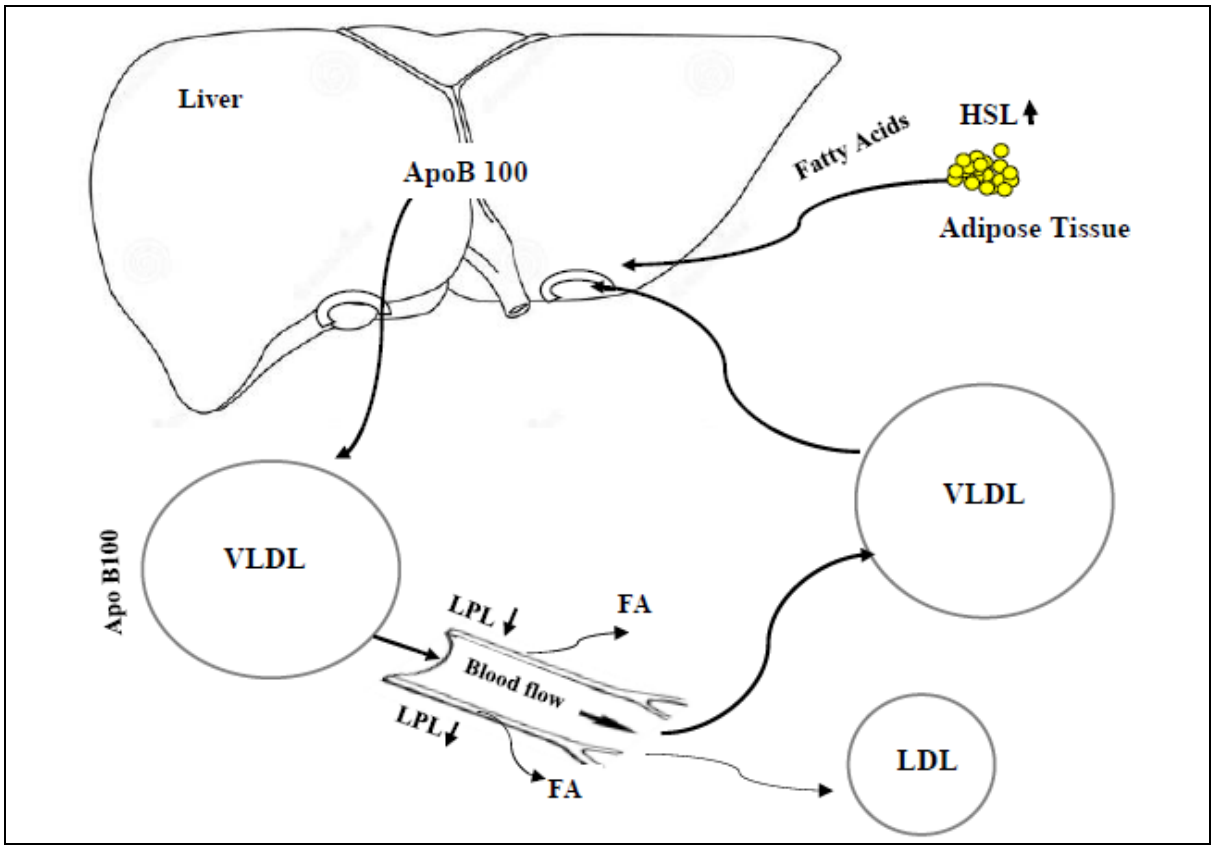


Figure 1.5: Schematic representation on the mechanism of VLDL production in diabetes. FA – Fatty Acids; HSL – Hormone Sensitive Lipase; LDL – Low Density lipoprotein; LpL – Lipoprotein Lipase; VLDL – Very Loew Density Lipoprotein.

Patients with poor glycaemic control have increased plasma levels of VLDL because the deficiency of insulin reduces its suppression of HSL resulting in increased release of fatty acids from adipose tissue into the circulation (Meijssen et al., 2001). The fatty acids returning to the liver are reassembled into TG and incorporated into VLDL. Like chylomicrons, VLDL requires LpL for its plasma catabolism, leading to the production of LDL. The reduced level of insulin decreases the actions of LpL and therefore, less fatty acid is removed from VLDL. This causes large lighter VLDL to stay in circulation, returning to the liver without complete

conversion to LDL. This effect can also be seen in the LpL loss of function mutation (Hoffmann et al., 2000). Figure 1.6 below shows how the lipoproteins develop in DM giving rise to the triad of diabetic dyslipidaemia.

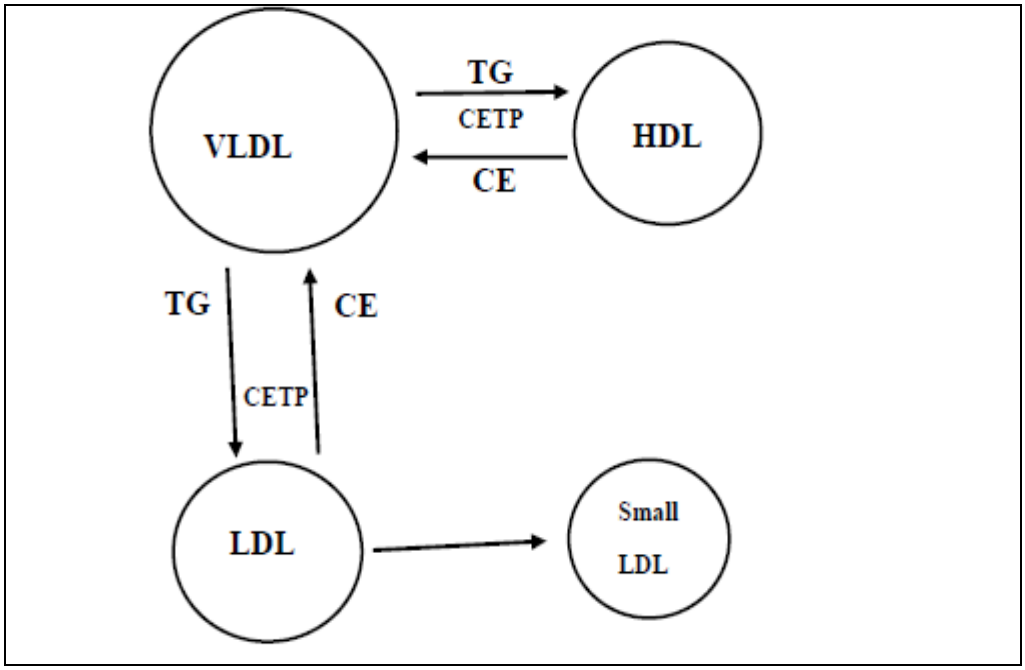


Figure 1.6: Schematic representation on the mechanism on the formation of HDL, LDL and VLDL in DM
CE – Cholesterol ester; CETP – Cholesterol Ester Transferase Protein; TG – Triglycerides; LDL – Low Density lipoprotein; VLDL – Very Low Density Lipoprotein

With increased concentrations of VLDL in the circulation, CETP will exchange VLDL triglyceride for cholesteryl ester in the core of LDL and HDL. The TG is hydrolysed and converted to free fatty acids by the actions of plasma lipases, especially hepatic lipase. This results in a decrease in size and an increase in density of both LDL and HDL (Krauss, 2004). Increased concentrations of plasma VLDL drives the exchange of TG from VLDL for the cholesteryl esters found in HDL mediated by CETP (Hayek et al., 1993). Thus, HDL loses its cholesterol ester in exchange for TG. It is the TG in HDL and not cholesteryl ester, which is a substrate for hydrolysis by plasma lipases, especially hepatic lipase resulting in a smaller HDL that is more rapidly cleared from the plasma resulting in low HDL measurement (Horowitz et al., 1993). Secondly in clinical laboratory measurements, it is the cholesterol component that is measured and therefore, substitution of TG for CE in the core of the particle leads to a decrease in this measurement.

1.10 Problem Statement

The third world/developing world paid more attention to infectious diseases until it started to become evident that non-communicable diseases were emerging as the leading cause of morbidity and mortality. Like in other developing countries non-communicable diseases appeared to manifest in the African people after migration to urban areas, a feature that is affecting the whole continent (Kinra et al., 2011). DM is by far the most common non-communicable disease with cardiovascular disease, a macrovascular complication of DM as a leading cause of death. A person with DM is at greater risk of developing heart attack than a person without. Dyslipidaemia has been identified as one of the factors associated with progression and development of cardiovascular disease in DM. It has also been seen that not all people with DM develop dyslipidaemia. The dyslipidaemia that develops has largely been labelled as secondary dyslipidaemia. Thus, we seek to contribute knowledge on the possible role of ApoE and PCSK9 genetics in the development of diabetic dyslipidaemia in the African population. The absence of dyslipidaemia in some diabetic patients as well as differences in the pattern of dyslipidaemia in the diabetic patients suggests the possible presence of an additional factor.

1.10.1. Research Question

What are the roles of ApoE and PCSK9 genetic variations in the development of dyslipidaemia in diabetic patients of African descent?

1.10.2. Hypothesis

1.10.2.1 Null hypothesis

Apolipoprotein E polymorphism and PCSK9 variants do not have a role in the type or form of dyslipidaemia that develops in diabetic patients of African descent.

1.10.2.1 Alternative Hypothesis

Different ApoE and PCSK9 variants influence the type or form of dyslipidaemia that develops in diabetic patients of African descent.

1.10.3. Broad Aim and Objectives

The broad aim of this study was to investigate the effects of genetic variation in apolipoprotein E and Proprotein convertase subtilisin/kexin type 9 in the development of dyslipidaemia in diabetic patients of African descent.

The aim was met through the following objectives:

1. Recruitment of diabetic dyslipidaemic and non-dyslipidaemic participants.
2. Determine the ApoE and PCSK9 distribution between dyslipidaemic and non-dyslipidaemic participants.
3. Establishing the presence and phenotype characterisation of dyslipidaemia.
4. Correlation of ApoE genotypes with different lipoprotein profile phenotypes.
5. Correlation of PCSK9 with different lipoprotein profile phenotypes.
6. Simple assessment of possible Gene-Gene (ApoE-PCSK9) Interactions.

CHAPTER 2: MATERIALS AND METHODS

2.1 Ethical clearance

The study was granted ethical clearance by both University of Cape Town, Human Research Ethics Committee (Ref: 089/2013) and University of Witwatersrand Ethics Review Committees (Ref: M130130) (Appendix Ai and Aii).

2.2 Study site

The study was conducted in the Diabetic Clinic and Chemical Pathology Department of Chris Hani Baragwanath Academic Hospital (CHBAH) located south of Johannesburg in South Western Townships (SOWETO). CHIBAH is the third largest hospital in the world occupying 70 ha (170 acres), with 3 200 beds and 6 760 staff members. It is a referral centre for 34 polyclinics and clinics, and one district hospital in SOWETO.

2.3 Study population

The study population consisted mainly of diabetic patients referred from clinics in Soweto and other medical centres. They were all of Bantu origin, comprising several ethnic groups from around southern, central and east Africa.

2.3.1 Study Design

The study was a mixed design which used a respective cohort, whose clinical data informed on invitation to participate. Participants were then purposively recruited into either dyslipidaemic or non-dyslipidaemic groups. Patients with specific dyslipidaemic patterns were included in the dyslipidaemic group and were further grouped into three: hypercholesterolemia (TC); hypertriglyceridemia (TG) and mixed profile (TC+TG).

2.3.2 Sample size determination

The required sample size was determined by the following equation: $n = [Z^2 (100\text{-}p)\, pd] \div \mathcal{E}^2$

where n = required size number

Z = Critical value

P = proportion, according to a recent paper (Daya et al., 2017), the proportion of dyslipidaemia among T2DM patients is approximately 94%.

Ɛ = Margin of error (we decided on a margin of error of 5%)

d = design effect, in our case will be 2 phenotypes (dyslipidaemia and non-dyslipidaemia).

Z-score corresponds to a confidence level which is a constant value needed for this equation.

Therefore,

$n = [Z^2 (100-p) pd] \div Ɛ^2$

$n = [1.96^2 (100-94) 94x2] \div 5\%^2$

n= [3.8416 x 6 x 184] ÷ 25

n = 173.

The minimum sample size required was 173 participants. Two hundred and forty-four (n=244) participants were recruited from CHBAH. A larger sample size ensured that power of the study was not lost in the event of some samples yielding poor DNA quality and quantity.

2.3.3 Sampling Technique

The study recruited patients from a retrospective cohort of diabetic patients. A simple purposive sampling technique was used to select patients with any of the dyslipidemia defined by TC>5.2mmol/L, TG>1.7mmol/L, both TC and TG high) and non-dyslipidemia defined by TC<5.2mmol/L and TG<1.7mmol/L. Participants were recruited in the ratio of 2 dyslipidaemic participants to 1 non-dyslipidaemic participants respectively.

2.3.4 Recruitment of dyslipidaemic participants

Self- confirmed black African patients of Bantu origin with ancestral information to three generations were recruited in the diabetic clinic at CHBAH. Participants were selected from those with full result profiles of laboratory confirmed dyslipidaemia. Patients of African Bantu Ancestry greater than 18 years of age with dyslipidaemia after diagnosis of T2DM who were willing to consent got included in the study. In addition, availability of medical records and DNA after extraction was also part of the inclusion criteria. Exclusion criteria included**:** patients with known primary causes of dyslipidaemia; patients with non-diabetic related

secondary cause of dyslipidaemia such as thyroid disorders; patients with liver failure; diabetic patients with prior renal pathology; pregnant patients; patients with DNA samples of low integrity and patients with missing medical records.

2.3.5 Recruitment of non-dyslipidaemic participants

Initially we looked for 'healthy' hospital workers to be the non-dyslipidaemic participants, but abandoned the idea after noticing that 4 out of the first 10 volunteers fell in either impaired fasting glycaemia (IFG), or impaired glucose tolerance (IGT) or were unaware that they had DM. Using the exclusion rule for the study, we recruited participants with good control of DM without any form of apparent dyslipidaemia. Recruitment within the same environment was due to the assumption that the distribution of exposure among the non-dyslipidaemic participants was the same as in the dyslipidaemic participants. All the non-dyslipidaemic had normal HbA1c and normal lipid profiles and was not on any lipid lowering medications.

2.4 Analytical methods

2.4.1 Glycated Haemoglobin (HbA1c)

2.4.1.1 Point of Care (Diabetic Clinic) Measurement of HbA1c)

In the diabetic clinic, DCA Vantage is used as Point of care testing (POCT) analyser utilising capillary blood samples (Appendix Bi). The analyser employs inhibition of latex agglutination for the measurement of specific HbA1c. An agglutinator (synthetic polymer containing multiple copies of the immunoreactive portion of HbA1c) causes agglutination of latex coated with HbA1c specific mouse monoclonal antibody. This agglutination reaction causes increased scattering of light, which is measured as an increase in absorbance at 531nm wavelength. HbA1c in whole blood specimens competes for the limited number of antibody-latex binding sites causing an inhibition of agglutination and a decreased scattering of light. The decreased scattering is measured as a decrease in absorbance at 531nm. The HbA1c concentration is then quantified using a calibration curve of absorbance versus HbA1c concentration. The percentage of HbA1c in the sample is then calculated as follows: % HbA1c = ([HbA1c] / [Total Haemoglobin]) x 100. Details for this method are in Appendix Bi and assay key performance data is included in Table 2.2. DCA Vantage was supplied by

Siemens in Rosebank, Johannesburg, South Africa. In the event of any discrepancy or during stock out, diabetic clinic patients' venous samples were tested in the central laboratory.

2.4.1.2 Central Laboratory Measurement of Glycated haemoglobin (HbA1c)

In the Central Laboratory, samples for HbA1c measurement are collected as whole blood in Ethylenediamine tetra acetic acid (EDTA) tubes. The sample is haemolysed by a haemolysing reagent that incorporates tetradecyl trimethyl ammonium bromide (TTAB) as the detergent to eliminate interference from leukocytes. All haemoglobin variants which are glycated at the β-chain N-terminus and, which have antibody-recognisable regions identical to that of HbA1c are determined by this assay. Therefore, the metabolic state of diabetic patients having uraemia or the most frequent hemoglobinopathies (HbAS, HbAC, and HbAE) can be determined by this assay.

HbA1c measurement was done on two different platforms. For diabetic in-patients and those admitted through the medical admission ward, the measurement was done in the Central Laboratory on a Roche analyser Cobas Integra 400. On the Cobas integra 400, haemoglobin A1c determination is based on the turbidimetric inhibition immunoassay (TINIA) for haemolysed whole blood. Haemoglobin A1c (HbA1c) in the sample reacts with anti-HbA1c antibody in the first reagent to form a soluble antigen-antibody complex. The polyhaptens in the second reagent react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex (Excess anti-HbA1c antibodies + Polyhaptens → Antibody-polyhapten complex) that is determined turbidimetrically. The percentage of HbA1c calculation utilises a dual but sequential method in which both Hb and HbA1c are measured in the same cuvette. The liberated haemoglobin in the haemolysed sample is converted to a stable derivative that is measured photometrically during the initial phase of the immunological reaction. In the subsequent reaction, glycohemoglobin (HbA1c) in the sample reacts with the anti-HbA1c antibody to form soluble antigen-antibody complexes. The two separate measurements are expressed as mmol/mol HbA1c according to International Federation of Clinical Chemists (IFCC), which is a ratio or % HbA1c according to Diabetes Complications and Control Trial (DCCT)/National Glycohemoglobin Standardisation Program (NGSP). They are calculated from the (HbA1c/Hb) x 1,000 formula under the IFCC and the units are mmol/mol and (HbA1c/Hb) x 91.5 + 2.15 formula for NGSP/DCCT with units expressed as a percentage (%) (Appendix Bii).

In preparation for this project, correlation studies between the two analysers using two possible sample types: capillary samples for patients in the diabetic clinic run on the DCA Vantage and venous samples for in-patients and those admitted through the medical admission ward were conducted. The rationale for sample type correlation was that most POCT tests on blood use capillary blood but discrepancies or confirmation for POCT results are resolved in the central laboratory that uses venous blood (Tanyanyiwa et al., 2015).

2.4.2 Lipid Profile Measurements

Tests that comprise a lipid profile in most laboratories include TC, TG, and HDL and LDL. Measurements of lipid profiles were done using a Roche Modular diagnostic platform supplied by Roche Diagnostics (Randburg, South Africa).

2.4.2.1 Total cholesterol

TC was measured using the fully automated enzymatic method (Appendix C) and the key assay performance data is included in Table 2.2 below. The principle employed is based on the enzymatic cleavage of CE by cholesterol esterase. The conversion of TC to cholest-4-en-3-one and hydrogen peroxide is catalysed by cholesterol oxidase. The hydrogen peroxide forms the quinonemine dye in the Trinder reaction. Other assays like those utilised on some Beckman Instruments do not incorporate the Trinder reaction step; they determine TC concentration by amperometric measurement of the rate of oxygen consumption. CE are cleaved by the action of cholesterol esterase to yield UC and free fatty acids. Total cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide forms a red colour after reacting with 4-aminophenazone and phenol (Trinder reaction).

The colour intensity is directly proportional to the concentration of TC. The testing principles for TC are encapsulated by the three equations below:

1) Cholesterol Esters (CE) + H_2O $\rightarrow$ Cholesterol + Fatty Acids

2) Cholesterol + Oxygen $\rightarrow$ Cholest-4-en-3-one + H_2O_2

3) $2H_2O_2$ +4-aminophenozone + phenol $\rightarrow$ 4-p-benzoquinone-monoimino)- phenazone + 4 H_2

2.4.2.2 High Density Lipoprotein (HDL)

High Density Lipoprotein (HDL) is measured using the homogeneous enzymatic colorimetric test (Appendix II). In the presence of magnesium ions, dextran sulphate selectively forms water-soluble complexes with low density lipoprotein (LDL), very low-density lipoproteins

(VLDL) and chylomicrons, which are resistant to polyethylene glycol (PEG) -modified enzymes. The cholesterol concentration in HDL is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40 %). Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase and in the presence of oxygen, cholesterol is oxidised by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide as shown below;

(a) HDL-cholesterol esters + $\xrightarrow{\text{PEG-cholesterol esterase}}$ HDL-cholesterol + H_2O + RCOOH

(b) HDL-cholesterol + $\xrightarrow{\text{PEG-cholesterol oxidase}}$ Δ4-cholestenone + O_2 + H_2O_2

(c) 2 H_2O_2 + 4-amino-antipyrine + peroxidase → purple-blue pigment + HSDA* + H^+ + H_2O + 5 H_2O

(where *HSDA = Sodium N-(2-hydroxy-3-sulfopropyl) −3,5-dimethoxyaniline).

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The colour intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically.

2.4.2.3 Triglycerides (TG)

The determination of TG is comprehensively described in Appendix III but key assay performance data is included in Table 2.1 below. The principle employed is based on the lipase-catalysed hydrolysis of TG to gylcerol and fatty acids. The glycerol portion of the TG molecules is used to determine TG concentration after hydrolysis. The TG measurement method is based on the principles below. In this method, TG is hydrolysed by lipase to release glycerol and fatty acids. The glycerol is reacted with adenosine triphosphate (ATP) in a reaction catalysed by glycerol kinase with magnesium as a co-factor to produce glycerol-3-phosphate and adenosine diphosphate (ADP). The ADP is reacted with phosphoenolpyruvate in a reaction catalysed by pyruvate kinase to produce ATP and pyruvate. The pyruvate then reduces NADH to NAD + lactate in a reaction catalysed by lactate dehydrogenase.

1) TG + 3 H2O → glycerol + 3 Fatty acids (RCOOH)

2) Glycerol + ATP → glycerol-3-phosphate + ADP

3) Glycerol-3-phosphate + O_2 → dihydroxyacetone phosphate + H_2O_2

4) H_2O_2 + 4-aminophenazone + 4-chlorophenol 4-(p-benzoquinone-monoimino)-phenazone + 2 H_2O_2 + HCl.

2.4.2.4 Low Density Lipoprotein (LDL)

Low Density Lipoprotein (LDL) can be measured using both direct and indirect methods. The indirect method based on the Friedewald Equation to calculate LDL using an empirical

equation, LDL = (TC – HDL– (TG/2.22) for units in mmol/L was used for this project. The factor, TG/2.22 is an estimate of VLDL concentration and is based on the average ratio of TG to TC in VLDL (Friedwald et al., 1972). The indirect or calculated method assumes that TC is composed of VLDL, LDL and HDL (i.e. TC = VLDL + LDL +HDL). The disadvantage of this method is that at high TG levels >4.5mmol/L samples contain chylomicrons, which have higher TG/cholesterol ratios than normal. Therefore, the use of the Friedewald equation would overestimate VLDL resulting in underestimating LDL. The opposite error occurs if the equation is used for patients with Type III hypercholesterolaemia because VLDL has a TG/cholesterol ratio in the order of 3:1 and therefore, application of the factor would underestimate VLDL resulting in the overestimation of LDL. To overcome this problem, the direct LDL method is normally done for diagnostic purposes. Quality Control of lipid measurements was carried out using internal and external quality-control schemes that are used to ensure high accuracy and precision in these determinations. For internal quality control, Precinorm and Precipath control sera were used and results of the tests were only accepted when the results for control sera were within the manufacture's given range. For external quality control, unknown concentration control sera from the BIORAD cycle quality control program were assayed, and the results were within two standard deviations ($\pm$2 SD) during the period our samples were run.

The central laboratory (site of analysis) participates in Royal College of Pathologists of Australasia (RCPA) external quality control programmes and is South African National Accreditation System (SANAS) accredited. A summary of the basic mandatory assay performance information is shown in Table 2.1 below.

Table 2.1 Summary of key assay performance information

Assay	**Analytical measuring range**	**Wavelength**	**CV**
TC	0.1 – 20.7 mmol/L	(sub/main) 700/505 nm	1.6%
TG	0.1– 10.0 mmol/L (has 1:5 auto dilution)	(sub/main) 700/505 nm	1.9%
HDL-C	0.08– 3.12 mmol/L	(sub/main) 700/600 nm	1.5%
HbA1c	4.3– 9.5 %	A/B 378/659 nm	1.3%
HbA1c (POCT)	2.5% – 14%	531 nm.	3%

CV – coefficient of variation; HDL – High density lipoprotein; TC – Total cholesterol; TG – Triglyceride; nm – nanometres.

2.5 Genotyping

2.5.1 DNA Extraction and Quality Control

DNA was extracted from whole blood samples using the salting out method according to Gustafson et al., (1987) with minor modifications (Appendix F). Extracted genomic DNA was quantified using the Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Genomic DNA intactness and integrity were verified on a 1% agarose gel stained 5µL/mL of the nucleic acid stain GR Green (Excellgen, Rockville, Maryland, USA). The molecular weight marker used to guide the size of the DNA was Gene Ruler TM 100bp plus ladder (Thermo Fisher Scientific, Wilmington, DE, USA). Electrophoresis proceeded at 80V for an hour. After electrophoresis the DNA bands were visualised using the Fire Reader V4 Vu Gel documentation system (UVitec Limited, Cambridge, UK).

2.5.2 Genetic characterization of ApoE

ApoE gene variation has been shown to play a role in the differences in lipid levels observed among patients. We investigated the role of variants E2, E3 and E4 among South African patients.

2.5.2.1 PCR for ApoE

ApoE gene was amplified using forward and reverse primers 5'-TCC AAG GAG CTG CAG GCG GCG CA-3' and 5'-GCC CCG GCC TGG TAC ACT GCC A-3' respectively. Primers were adapted from a method designed by Marrzoq et al., 2011. A 25µL reaction consisting of 50ng/µL DNA template, 1U Dream Taq Polymerase (ThermoScientific, Waltham, USA), 0.2mM dNTPs (Kapa Biosystems, Cape Town, South Africa), 0.4 µM of each primer (Integrated DNA Technologies, Illinois, USA), 5x Go Taq flexi buffer (ThermoScientific, Waltham, USA) and 1.5 mM magnesium chloride (ThermoScientific, Waltham, USA) made up to 25µL using nuclease free distilled water (ThermoScientific, Waltham, USA). Amplification was done on the Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, California, USA), the reaction conditions were an initial denaturation of 95 °C for 3 minutes, followed by 40 cycles of denaturation of 95°C for 60 seconds, annealing at 64°C for 30 seconds and an extension at 72°C for 2 minutes. Final extension was 72°C for 10 minutes. PCR products were analysed on 2 % agarose gels to check specificity and size of the products. The amplicon was 218 base pairs in length.

Determination of the different ApoE alleles was done according to previously established. Restriction enzymes AflIII and HaeII purchased from Thermo Fisher Scientific, USA were used to digest the amplified ApoE gene fragment.

2.5.2.2 Digestion with AflIII and HaeII for ApoE genotyping

Two ApoE single-nucleotide polymorphisms (SNPs), *rs429358C>T* at position 112 and *rs7412C>T* at 158 were examined in this project. They are found in exon 4 of ApoE gene where they produce missense mutations. Digestion with *AflIII* to genotype rs429358 was achieved due to the enzyme property of cutting whenever a T allele appears producing 168bp and 50bp fragments. If a C allele is present, the cut site is abolished and 218bp intact fragment results. Digest with HaeII to genotype rs7412 was achieved due to the enzyme property of cutting whenever a C allele appears producing 195bp and 23bp fragments. If a T allele appears, the cut site is abolished resulting in a 218bp intact fragment.

To genotype rs7412 and rs429358 single nucleotide polymorphisms, 10 uL of PCR products were digested overnight at 37°C with *Hae*II and *AflI*II, respectively. The digested product was then electrophoresed on 3.5% agarose at 60V for 6 hours. The PCR-RFLP results were validated Sanger sequencing on ABI 3130xl Genetic analyser (Applied Biosystems, California, USA). The expected enzyme digestion products of ApoE are shown in table 2.2 below.

Table 2.2 A*flI*II and *Hae*III enzyme fragmenting patterns

Haplotype	rs429358	rs7412	AflIII digest Fragment bp	HaeIII Digest Fragment bp
ApoE3	T	C	168	195
ApoE2	T	T	168	218
ApoE4	C	C	218	195

2.5.3 Characterisation of variation in PCSK9 gene.

PCSK9 variant PCSK 946L (rs11591147) on exon 1, I474V (rs562556) in exon 9 and E670G (rs505151) in exon 12 were characterized to compare lipoprotein levels between male and

female carriers of the E670G variant (rs505151). PCSK9 rs28362286 has been reported in 3.7% black African women attending two antenatal clinics in Zimbabwe and was associated with a 27% reduction in LDL (Hooper et al., 2007). It would therefore, mean that the low cholesterol translates to reduced cardiovascular risk.

2.5.3.1 Amplification of region of interest

The regions of interest were on exon 12 of *PCSK9* gene and harbour the rs28362286 and rs505151 polymorphisms. The region containing PCSK9 rs505151was amplified using forward and reverse primers 5'-TCT CCT CGC CAG GAC AGC AAC CT-3'and 5'-TGA GGC CCG AGA GGA AAC AGC A-3' respectively. A 25µL reaction consisting of 50ng/µL DNA template, 1U Dream Taq Polymerase (ThermoScientific, Waltham, USA), 0.2mM dNTPs (Kapa Biosystems, Cape Town, South Africa), 0.4 µM of each primer (Integrated DNA Technologies, Illinois, USA), 5x Go Taq flexi buffer (ThermoScientific, Waltham, USA) and 1.5 mM magnesium chloride (ThermoScientific, Waltham, USA) made up to 25µL using nuclease free distilled water (ThermoScientific, Waltham, USA). Amplification was done on the BioRad T100 Thermal Cycler (Bio-Rad Laboratories, California, USA), the reaction conditions were an initial denaturation of 95 °C for 3 minutes, followed by 34 cycles of denaturation of 95°C for 30 seconds, annealing at 57°C for 30 seconds and an extension at 72°C for 30 seconds. Final extension was 72°C for 5 minutes. PCR products were analysed on 2 % agarose gels to check specificity and size of the products.

The amplification steps for region containing PCSK9 rs28362286 were the same as described above for PCSK9 rs505151 except that denaturation was followed by annealing at 54ºC for 30 seconds. The following forward and reverse primers 5'-TGT CGG AGG GAG AAA TGA AGT GT-3'3'and reverse primer 5'-TGG AGG GCT GAG AGA GGG AGA-3' were used for amplification of the region containing PCSK9 rs28362286 respectively.

2.5.3.2 Post PCR Clean up

PCR products were purified to remove excess dNTPs and unincorporated primers using a standard method which includes the combined use of Exo1 and FastAp (Appendix A17). 1U of FastAp™ Thermosensitive alkaline phosphatase (Thermo Scientific, Waltham, USA) and 4U of Exonuclease 1 (Exo1, ThermoScientific, Waltham, USA) was added to 5µL of the PCR product and the reaction made up to 20µL using nuclease free distilled water. The clean-

up reaction proceeded at 37°C for an hour followed by inactivation of both enzymes at 75 °C for 15 minutes. The entire clean up reaction was done in the BioRad MyCycler Thermal cycler.

2.5.3.3 Sanger sequencing reaction

Sequencing of the PCR products was done using the BigDye® Terminator v3.1 cycle Sequencing Kit (Life Technologies, California, USA). The sequencing reaction involves cycles of denaturation of the template PCR product followed by annealing of the sequencing primer and extension of the primer by DNA polymerase with the random incorporation of ddNTPs. Two sequencing reactions were set up per each PCR product: one with a forward sequencing primer and the other with a reverse sequencing primer. The sequencing primers were the same as the PCR primers. Post sequencing precipitation of DNA was done using a standard ethanol precipitation coupled with salting out of proteins using sodium acetate. Capillary electrophoresis of sequencing products was done on the ABI 3130*x*l Genetic Analyser (Applied Biosystems, California, USA).

2.6 Data Analysis

2.6.1 Sanger Sequencing data analysis

Sequencing data visualisation, assembly and analysis was done using SeqMan Pro (DNASTAR Inc., Madison, USA) software. Alignment of sequences was done against reference sequences accessed from the NCBI Gene website (https://www.ncbi.nlm.nih.gov/gene).

2.6.2 Allele and Genotype Frequency Distribution

Frequency distribution of alleles was determined and adherence to Hardy Weinberg theorem checked. The Hardy-Weinberg Equation theorem states that allele frequencies in a population will not change from generation to generation if the breeding population is large; mating is random; there are no mutations; no emigration and immigration and no natural selection.

2.6.3 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Version 5, GraphPad Software Inc. San Diego, CA) and STATA (Version 11, Stat Soft, USA) statistical programs. Normality was tested using Shapiro-Wilk Test. Generally, nonparametric tests were used to analyse the data. Mann Whitney or Kruskal-Wallis (with Dunn's Multiple Comparison) tests were used to assess association between clinical parameters, or genotypes for each SNP and

lipoprotein levels/concentrations. Statistical significance for the univariate analysis was defined as $p < 0.05$. Two sample t-test was used to compare distribution percentage values between dyslipidaemic and non-dyslipidaemic participants. A one-sample t-test was used to compare the difference in percentages within the same group. In both cases statistical significance was also defined as $p < 0.05$. To assess dyslipidemia produced by the two PCSK9 genotypes in relation to each of the ApoE genotypes, medians of the different measured parameters for the two PCSK9 variants were compared using t-test nonparametric statistics to assess the dyslipidemia.

CHAPTER 3: RESULTS

3.1 Demographic characteristics of the cohort

The study subjects were divided into two groups dyslipidaemic (n =165) and non dyslipidaemic (n =79) based on their lipid profiles. The non-dyslipidaemic participants were patients with good glycaemic control and without dyslipidaemia. The 165 dyslipidaemic participants were further divided into three groups; those with high TC (33.3%, n=55), those with high TG (29.1%, n=48) and those with both high TC and TG (37.6%, n= 62). The cohort comprised of 128 (52%) females and 116 (48%) males. The characteristics of the participants are shown in Table 3.1 below.

Table 3.1: Demographic Characteristics of the cohort

Parameter		Non Dyslipidaemic (n=79)	Dyslipidaemic (n=165)	p-value
Age	Median (IQR)	51.00 (42.0 –60.0)	59.00 (52.0 – 64.0)	**<0.0001**
Systolic blood pressure	Median (IQR)	133.0 (125 – 147)	142.0 (127.5 –152.5)	0.0507
Diastolic blood pressure	Median (IQR)	79.0 (74.0 – 87.0)	81.0 (74.0 – 89.0)	0.9684
Weight (kg)	Median (IQR)	75.0 (67.0 – 90.0)	82.00 (73.0 – 91.0)	**0.0018**
Height (metres)	Median (IQR)	1.61 (1.57 – 1.65)	1.61 (1.56 – 1.68)	0.9415
Body Mass Index	Median (IQR)	27.8 (26.2 – 29.2)	30.8 (27.05 – 35.35)	**0.0001**
HbA1c (%)	Median (IQR)	5.4 (4.78 – 5.90)	6.9 (5.85 – 10.85)	**0.0001**
Total Cholesterol (mmol/L)	Median (IQR)	3.7 (3.01 – 4.29)	5.8 (4.95 – 6.46)	**0.0001**
Triglyceride (mmol/L)	Median (IQR)	1.0 (0.75 – 1.3)	2.2 (1.44 – 2.88)	**0.0001**
LDL-Cholesterol (mmol/L)	Median (IQR)	2.0 (1.53 – 2.53)	3.4 (2.64 – 4.21)	**0.0001**
HDL-Cholesterol (mmol/L)	Median (IQR)	1.2(0.91 – 1.45)	1.3(1.03 – 1.57)	**0.0104**
Non-HDL (mmol/L)	Median (IQR)	2.5 (2.03 – 2.98)	4.2 (3.71 – 5.19)	**0.0001**

Compared with non-dyslipidaemic participants, dyslipidaemic participants had significantly higher, body mass index, HbA1c (%), TC, TG, LDL, HDL and non-HDL ($p<0.05$). There was a slight tendency to significance (p-value= 0.051) in systolic blood pressure with dyslipidaemic participants being higher, but there was no difference in the diastolic blood pressure p- value = 0.9684. Age and height were significantly higher and in the dyslipidaemic ($p < 0.0001$) compared to non-dyslipidaemic participants ($p = 0.0018$) respectively. Height

between the two groups was not different (p =0.9415). The difference in the measured parameters between the dyslipidaemic and non-dyslipidaemic was expected because purposive sampling technique was employed. The characteristic features of the three dyslipidaemic groups, i) those with high TC only, ii) those with high TG only and iii) those with both high TC and TG were compared, and results are shown in Table 3.2 below.

Table 3.2 Demographic characteristics of the dyslipidaemic group

Parameter		High Cholesterol, N=55	High Triglyceride, N=48	Mixed, N=62	p-value
Age	Median (IQR)	59.00 (53.00-66.00)	57.00 (51.00-63.00)	60.0 (51.75 - 64.00)	0.9946
Systolic blood pressure	Median (IQR)	137.00 (126.0-152.0)	145.0 (133.3-156.3)	139.0 (126.3-152.0)	0.1125
Diastolic blood pressure	Median (IQR)	79.00 (73.0-88.0)	85.00 (77.0-90.0)	79.00 (73.0-85.5.0)	0.061
Weight (kg)	Median (IQR)	75.00 (64.0-91.00)	82.5 (76.25 -92.0)	83.50 (76.00 – 91.00	**0.0002**
Height (metres)	Median (IQR)	1.62 (1.56-1.68)	1.60 (1.56-1.68)	1.63 (1.57-1.68)	0.7943
BMI	Median (IQR)	27.70 (24.20-34.30)	31.75 (28.88 - 36.33)	31.20 (28.65-35.18)	**0.0023**
HbA1c (%)	Median (IQR)	6.60 (5.50-8.60)	8.65 (5.73-11.20)	6.95 (6.08-11.45)	0.166
Total Cholesterol (mmol/L)	Median (IQR)	6.15(5.68-6.61)	4.68 (4.13-4.92)	6.25 (5.75-6.80)	**<0.0001**
Triglyceride (mmol/L)	Median (IQR)	1.20 (1.03-1.45)	2.57 (2.34-3.13)	2.60 (2.10-3.41)	**<0.0001**
LDL-Cholesterol (mmol/L)	Median (IQR)	3.82 (3.23-4.49)	2.34 (1.55-2.65)	3.70 (3.02-4.30)	**<0.0001**
HDL-Cholesterol (mmol/L)	Median (IQR)	1.64 (1.40-1.99)	0.97 (0.77-1.20)	1.25 (1.06-1.43)	**<0.0001**
Non-HDL(mmol/L)	Median (IQR)	4.60 (3.80-5.19)	3.63 (3.24-3.90)	5.11 (4.40-5.67)	**<0.0001**

Similar to comparisons made between dyslipidaemic and non-dyslipidaemic participants, the differences in the measured parameters between the three groups (those with high cholesterol only, those with high triglycerides only and those with both high cholesterol and triglyceride /mixed) were also analysed. One-way analysis of variance (ANOVA) did not show any significant difference in HbA1c (p-value = 0.166) between the three groups. This means glycaemia/glycaemic control does not seem to influence the dyslipidaemic phenotype seen in DM. Unlike the significant difference (p <0.0001) in age between the dyslipidaemic and non-dyslipidaemic, there was no significant difference in age (p = 0.9947) between the three dyslipidaemic groups. The data shows significant difference (p = 0.0002) in weight. The mixed group had the highest weight with a median of 83.50kg (IQR 76.00 - 91.00) and the high cholesterol group had the lowest weight with a median of 75.00kg (IQR 64.0-91.00).

There was no significant difference in height, but BMI was significantly different (p =0.0023). These results show that triglyceride is the common factor associated with increased weight. Significant difference ($p < 0.0001$) between the lipoproteins in the three groups was expected because of purposive sampling technique used.

3.2 Associations between measured parameters in dyslipidaemic participants

In this analysis, each one of the measured analytes: HbA1c, TC, TG, LDL and HDL was analysed as a dependent variable against the selected cohort characteristics. A summary of the correlation analysis is summarised in tables 3.3 to 3.7 below. The study found significant association between HbA1c (table 3.3) and age. TC was strongly associated with its main fraction LDL, HDL, TG as well as Body Mass Index and HbA1c. TG was strongly associated with HbA1c and moderately associated LDL (Table 3.5). LDL (Table 3.6), the main cholesterol fraction was strongly associated with age, systolic blood pressure, diastolic blood pressure as well as HDL and HbA1c but moderately associated with gender. HDL was strongly associated with age (Table 3.7).

Table 3.3 Effects of patient characteristics on HbA1c

Measured parameter	OR	(CI) 95%	p values
Gender	0.88	(0.45- 1.74)	0.7704
Age	1.05	(1.02- 1.08)	0.0075
Systolic blood pressure	1.02	(0.99- 1.05)	0.1563
Diastolic blood pressure	0.99	(0.95 - 1.04)	0.9277
Weight	1.01	(0.99- 1.04)	0.2869
Height	9.13e-28	(0.00 - ∞)	0.9379

This analysis showed that HbA1c was strongly associated with age. Therefore, the older participants had poorer glycaemic control as reflected by higher HbA1c.

Table 3.4 Effects of patient characteristics on Total Cholesterol

Measured parameter	OR	(CI) 95%	p values
Gender	5.91e-02	(8.28e-01, 1.14)	0.78134
Age	9.73e-01	(9.96e-01, 1.01)	0.45877
Systolic blood pressure	1.0	(9.96e-01, 1.01)	0.57454
Diastolic blood pressure	1.0	(9.90e-01, 1.01)	0.94069
Weight	9.96e-01	(9.86e-01, 1.01)	0.49342
Height	4.69e-52	(1.26e-188, 1.75e+85)	0.53539
Triglyceride	1.12	(1.09, 1.15)	3.5e-10
Low Density Lipoprotein	2.34	(2.17, 2.52)	< 2e-16
High Density Lipoprotein	2.23	(1.87, 2.67)	1.5e-12
Body Mass Index	1.03	(1.01-1.06)	0.04931
HbA1c	1.06	(1.03-1.08)	0.00092

This analysis showed that Total cholesterol was strongly associated with low-density lipoprotein, high-density lipoprotein.
This is because both low-density lipoprotein and high-density lipoprotein are fraction of total cholesterol. There was a positive correlation between BMI and HbA1c with Total cholesterol respectively. Therefore, participants with a high BMI had poor glycaemic control and high cholesterol. This can be due to insulin resistance that develops followed by hyperglycaemia. High cholesterol is a result of the metabolic link between TC and TG that takes place in secondary dyslipidaemia.

Table 3.5 Effects of patient characteristics on Triglyceride

Measured parameter	OR	(CI) 95%	p values
Gender	0.60	(9.84e-01- 1.04e+00)	0.185
Age	1.01	(9.84e-01 - 1.04e+00)	0.5128
Systolic blood pressure	1.00	(9.81e-01- 1.03e+00)	0.7599
Diastolic blood pressure	0.99	(9.56e-01- 1.04e+00)	0.8367
Weight	0.98	(9.51e-01 - 1.02e+00)	0.5673
Height	4.18e+37	(0.00e+00 - ∞)	0.9076
Low Density Lipoprotein	0.72	(5.42e-01- 9.61e-01)	0.0612
High Density Lipoprotein	0.54	(2.71e-01- 1.08e+00)	0.1415
Body Mass Index	1.08	(9.67e-01- 1.20e+00)	0.2568
HbA1c	1.16	(1.05e+00-1.08e+00)	0.0175

This analysis showed that high triglyceride was strongly associated with high HbA1c. This is expected because insulin resistance is a common feature in T2DM. Fatty acids provide an alternative source of energy.

Table 3.6 Effects of patient characteristics on LDL

Measured parameter	OR	(CI]) 95%	p values
Gender	1.31	(1.029-1.67)	0.0654
Age	1.02	(1.004-1.03)	0.0174
Systolic blood pressure	1.01	(1.003-1.02)	0.0237
Diastolic blood pressure	0.98	(0.965- 0.996)	0.0397
Weight	0.99	(0.985-1.00)	0.1823
Height	1.66	(0.00, 2.93)	0.1814
High Density Lipoprotein	1.67	(1.286, 2.16)	0.0013
HbA1c	1.06	(1.017-1.10)	0.0184

LDL was strongly associated with most of the measured parameters such as older age, high systolic and diastolic blood pressure, HDL and HbA1c. Most of these parameters form part of the components that constitute metabolic syndrome. These results show why LDL has been implicated as the major player in cardiovascular pathology.

Table 3.7 Effects of patient characteristics on HDL

Measured parameter	OR	(CI) 95%	p values
Gender	0.9.7	(0.88-1.08)	0.6646
Age	1.01	(1.00-1.01)	0.0072
Systolic blood pressure	0.99	(0.99-1.00)	0.2991
Diastolic blood pressure	0.99	(0.99-1.01)	0.7636
Weight	1.00	(0.99- 1.00)	0.8924
Height	2.22e+43 4.81e+129]	(1.02e-43,)	0.4079
HbA1c	0.998	(0.98, 1.01)	0.81

With a p = 0.0072 the analysis showed that HDL is strongly associated age.

3.3 Genetic characterisation

3.3.1 DNA Quality Tests

The concentration and quality of the extracted DNA was analysed on the Nano drop spectrophotometer. The integrity of genomic DNA shown in Figure 3.1 below was assessed on a 1% agarose gel.

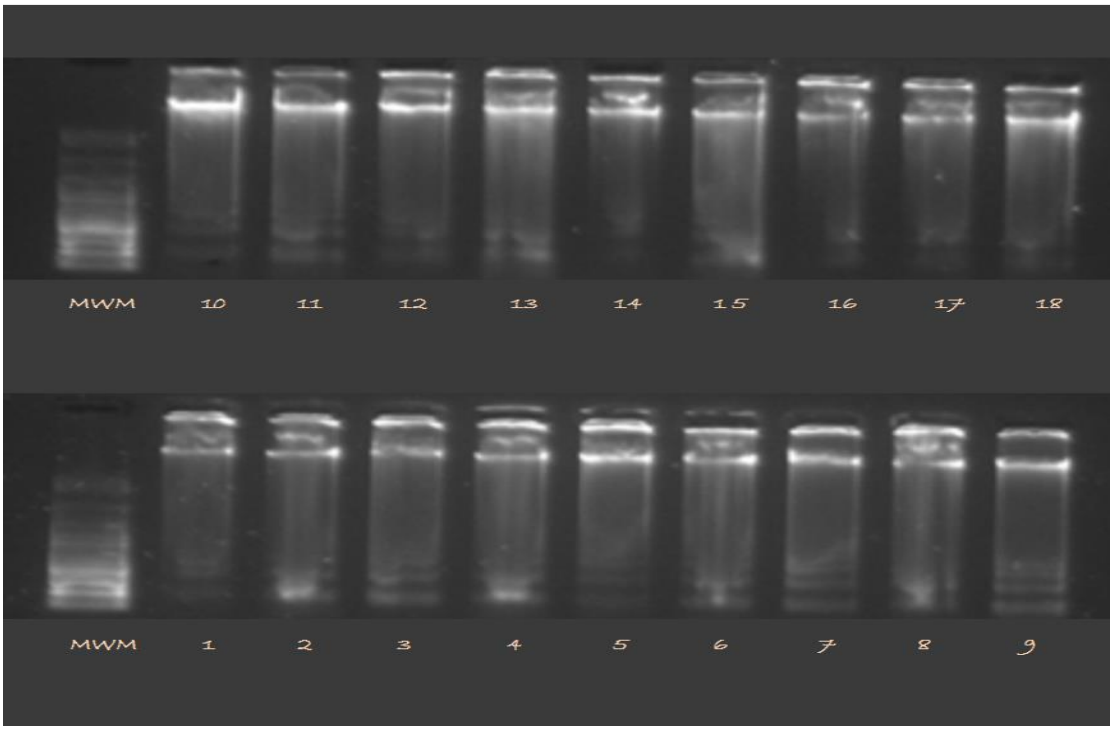
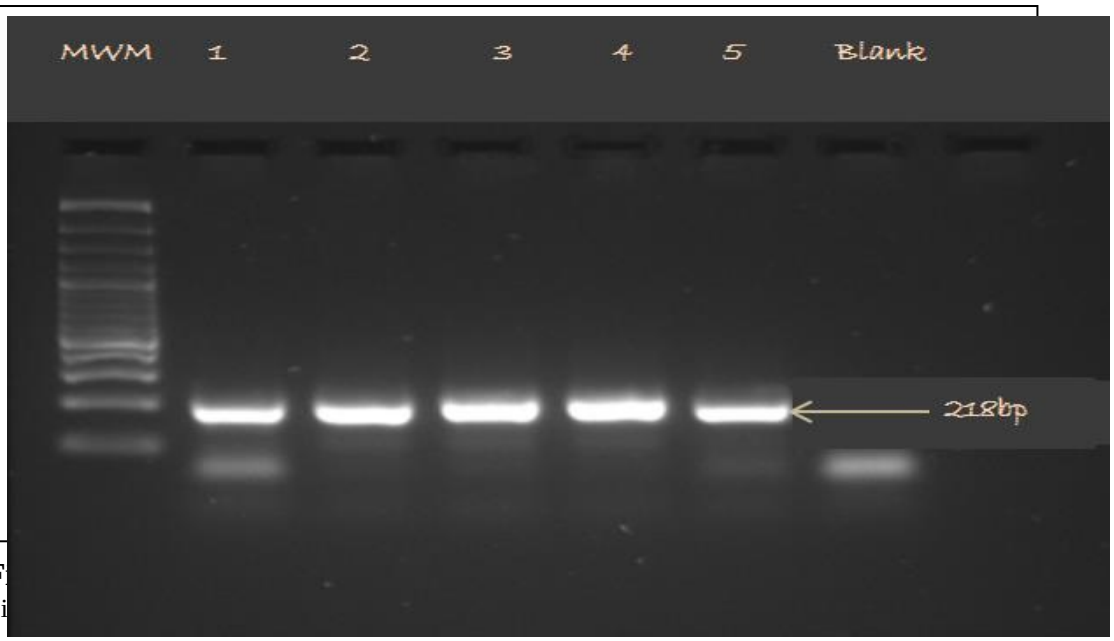


Figure 3.1 Assessing integrity of the genomic DNA
Agarose gel run for 1 hour at 5V/cm
MWM=molecular weight marker
Lanes 1 to 18 are DNA preparations from different patients

3.3.2 ApoE Amplification

After confirming the presence of genomic DNA, amplification of ApoE exon 4 encompassing the 218-single nucleotide polymorphism was performed as shown in Figure 3.2 below.



F SNP – Si

3.3.3 Identification of ApoE alleles

All six possible allele combinations including an unverified labelled in this project as ApoE X were identified after digestion with AflIII and HaeII enzymes. The digestion products were

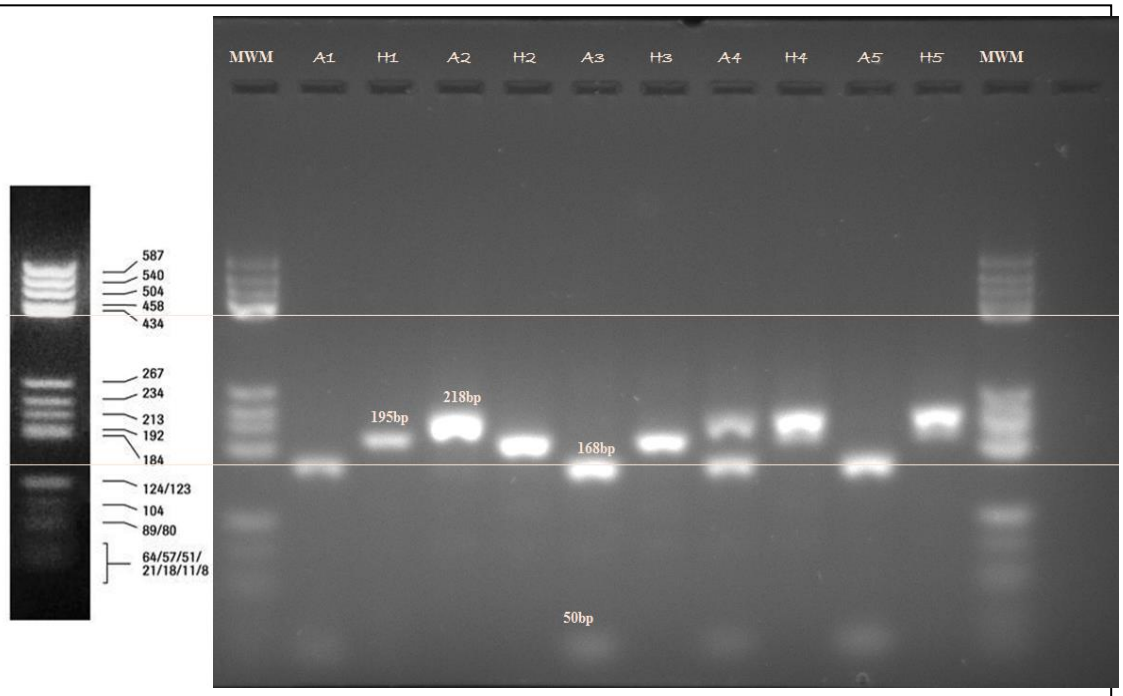


Figure 3.3 ApoE AflIII and HaeII Enzyme Digestion. Bp – base pairs; MWM-Molecular weight markers; A1 – A5 are DNA samples digested using AflIII

H1 – H5 are DNA samples digested using HaeII

Results of AflIII and HaeII digestion of ApoE electrophoresis on 3.5% agarose gel run for 6 hours at 60V. Sample 1= (A1 and H1): Sample 2= (A2 and H2): Sample 3 = (A3 and H3): Sample 4 = (A4 and H4) and sample 5 = (A5 and H5). The gel shows that HaeII identified the presence of rs7412C allele when ever the enzyme cuts the PCR product into twoone with 195bp and the other with 23bp. If T allele appears, the cut site is abolished leaving the 218bp fragment intact. On the other hand AflIII identified the presence of genotype rs429358 because it cuts whenever a T allele appeared giving rise to 168bp and 50bp fragments. If a C allele appears, the cut site is abolished and 218bp fragment was left intact.

3.4 PCSK9 Genetic characterisation

3.4.1 PCR for PCSK9

Of the 244 participants, 201 (138 dyslipidaemic and 63 non dyslipidaemic participants) were successfully genotyped for PCSK9. Results for the PCSK9 rs505151 PCR are shown in Figure 3.4 below.

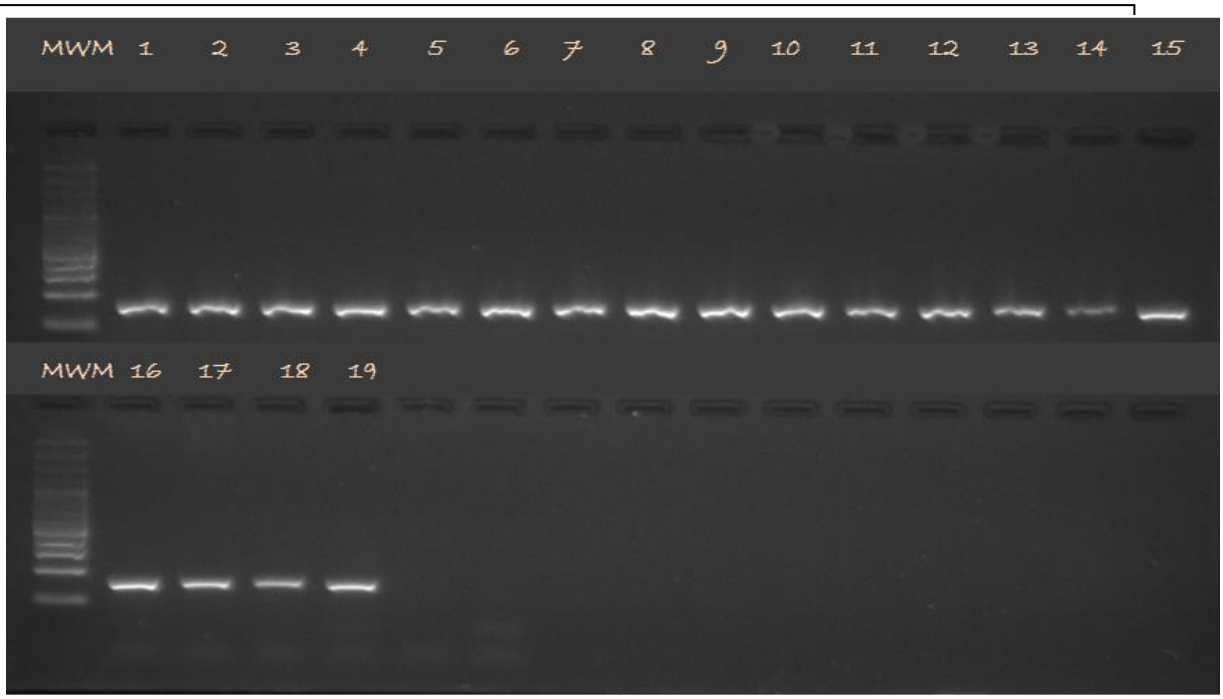


Figure 3.4: PCR for PCSK9 rs505151 SNP characterisation. PCR products were run on a 2% Agarose gel for 60 minutes at 100 Volts. MWM- Molecular weight markers. Lanes 1 – 19 are amplified DNA samples for PCSK9rs505151nt patients

The PCR expected product: 167 bp DNA fragment was clearly well defined in all the samples marked 1 to 19.

3.4.2 PCSK9 Sequencing Results

The sequencing results showing AA, AG and GG genotypes are shown in Figure 3.5 below.

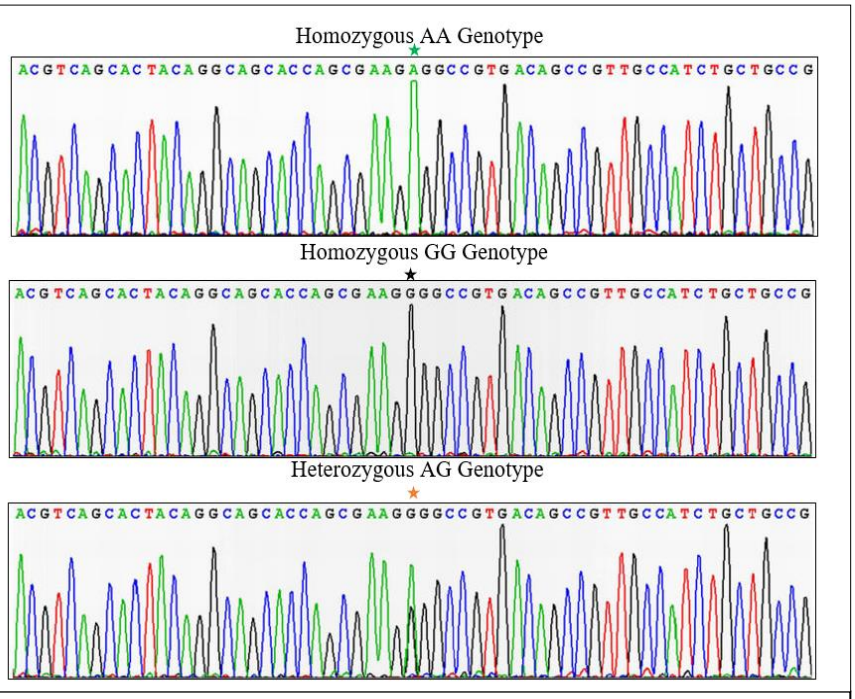


Figure 3.5 PCSK9 sequences chromatogram

The bases are colour coded A= green; C= purple, G= black and T= red Top. Homozygous produced a single peak with a single appropriate colour as seen in AA genotype (green) and GG genotype (black) in the top and middle frames respectively) Heterozygous produced two peaks which were superimposed at the same position with two different colours, each representing its own colour code as seen in heterozygous AG (green and black) in the lower frame.

3.5 ApoE rs429358 Genotype frequency distribution

Genotypes for ApoE conformed to Hard Weinberg equilibrium and we were able to detect all six common genotypes. In addition, there was a genotype with heterozygous features but could not be fully characterised and was labelled as ApoE X to differentiate it from the known genotypes. The frequency distribution of ApoE genotypes between the dyslipidaemic and non-dyslipidaemic participants were compared and shown in Table 3.4 below.

Table 3.8 Frequency of Apo E genotypes

Genotype	Dyslipidaemic n= 165	Genotype Frequency	Non Dyslipidaemic n= 79	Genotype Frequency	p-value
E2/2	10	0.059	4	0.05	0.775
E2/3	9	0.053	20	0.26	**<0.0001**
E2/4	3	0.018	0	0.00	0.231
E3/3	62	0.376	18	0.25	0.0522
E3/4	60	0.365	24	0.28	1.190
E4/4	6	0.035	2	0.03	0.839
ApoE X	15	0.082	11	0.03	0.839

There was a difference in the ApoE rs429358 genotype distribution between dyslipidaemic and non-dyslipidaemic participants. ApoE3/3 had the highest frequency in the dyslipidaemic participants, while being the third in the non-dyslipidaemic. ApoE2/3 ranked as the fifth in the dyslipidaemic but second in the non-dyslipidaemic subjects. ApoE2/4 was absent in the non-dyslipidaemic participants. ApoE3/3 carriers had slight tendency towards development of dyslipidaemia ($p = 0.052$). On the other hand, ApoE2/3 carriers showed significant resistance towards development of dyslipidaemia compared to other genotypes ($p<0.0001$).

3.6 PCSK9 variant frequency distribution

Genotypes for PCSK9 conformed to Hard Weinberg equilibrium and the study was able to detect all PCSK9 rs505151 and PCSK9rs28362286 genotypes targeted for the study. Table 3.5 below shows the distribution of the PCSK9 rs505151 and PCSK9 rs28362286 single nucleotide polymorphisms (SNP) and their variants.

Table 3.9 PCSK9 variant frequency Distribution

PCSK9rs 505151G>A	**Dyslipidaemic (n=138)**	**Genotype frequency**	**Non Dyslipidaemic (n=63)**	**Genotype frequency**	p-value
PCSK9rs505151G/G	6	0.04	6	0.10	0.095
PCSK9rs505151G/A	56	0.41	17	0.27	0.057
PCSK9rs505151A/A	76	0.55	40	0.63	0.178
PCSK9rs 28362286C>A	**Dyslipidaemic (n=138)**	**Genotype frequency**	**Non Dyslipidaemic (n= 63)**	**Genotype frequency**	
PCSK9rs 28362286C/C	130	0.94	63	1.00	<0.05
PCSK9rs28362286C/A	8	0.06	0	0.0	<0.05
PCSK9rs 28362286A/A	0	0	0	0.0	-

PCSK9 rs505151A/A had a high frequency distribution in both dyslipidaemic (55%) and non-dyslipidaemic (63%). There was no significant difference between the dyslipidaemic and non-dyslipidaemic ($p = 0.178$). PCSK9 rs505151G/A genotype frequency distribution in the dyslipidaemic (41%) had a slight tendency towards significance ($p = 0.057$) for development of dyslipidaemia compared to the non dyslipidaemic (27%). PCSK9 rs505151 G/G genotype was two-fold more in non-dyslipidaemic (10%) compared to dyslipidaemic (4%) participants but did not have any significance ($p=0.095$) towards resistance in development of dyslipidaemia.

PCSK9 rs28362286 C/C genotype had a notably high frequency distribution in both dyslipidaemic (94%) and (100%) in non-dyslipidaemic. PCSK9 rs28362286 C/A genotype had frequency distribution of 6% in the dyslipidaemic participants only and absent in non-dyslipidaemic participants. PCSK9 rs28362286 A/A genotype was absent in both dyslipidaemic and non-dyslipidaemic participants. There was a significant association ($p <0.05$) between diabetics carrying PCSK9 rs28362286 C/C and PCSK9 rs28362286 C/A respectively towards development of dyslipidaemia.

3.7 Comparisons of measured parameters between ApoE genotypes in dyslipidaemic participants.

In order to establish the influence of each ApoE genotype, comparison between each of the ApoE genotypes and the measured analytes: HbA1c, TC, TG, LDL and HDL was assessed, and the results are summarised in Figure 3.6 below.

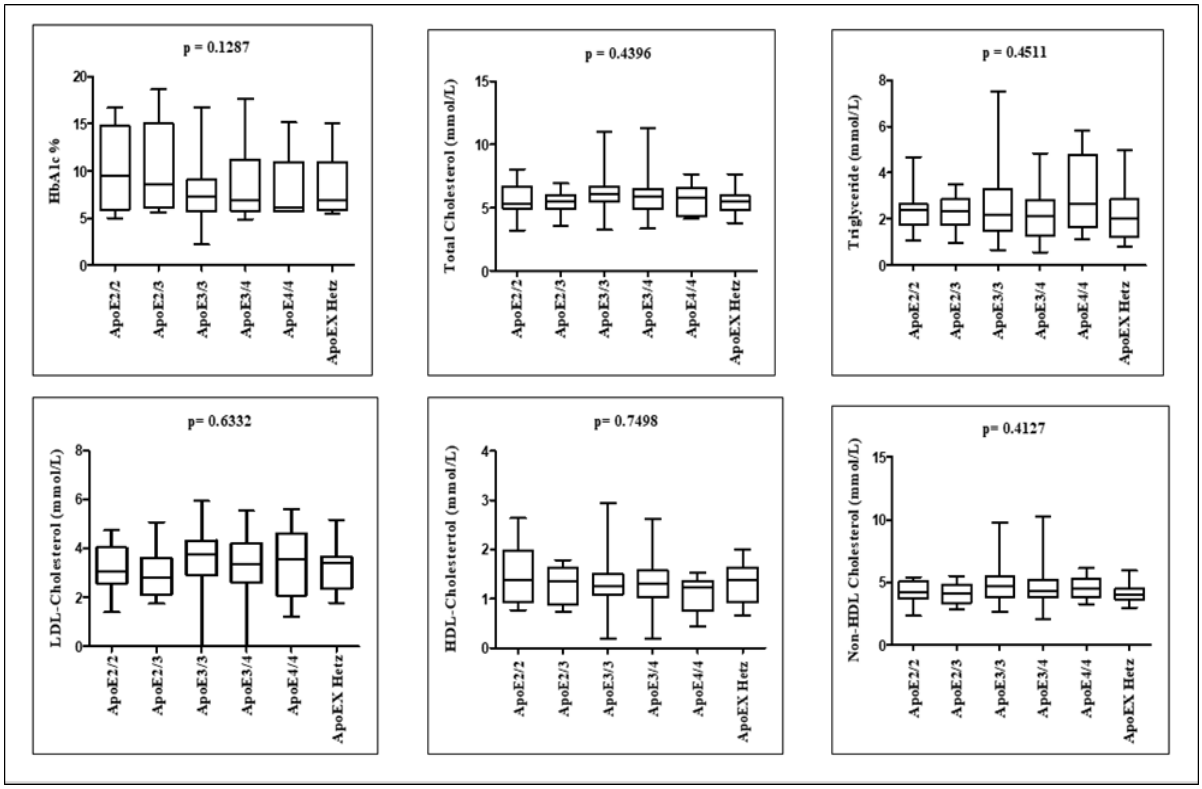


Figure 3.6: Influence of ApoE genotypes on measured parameters

The results show that carriers of the homozygous ApoE 2/2 genotypes had worst glycaemic control with a median HbA1c of 10.95% (IQR 5.88%-14.98%). Carriers of the homozygous ApoE 4/4 were shown to have the best glycaemic control with median HbA1c levels of 6.60% (IQR 5.70 – 12.3). In terms of lipoproteins; the results did not show any significant specific relationship between the ApoE genotypes to any pattern nor type of dyslipidaemia. Carriers of the homozygous ApoE 3/3 had the highest positive influence on TC with median of 6.06 mmol/L (IQR 5.48 – 6.71mmol/L). On the other hand, carriers of the homozygous ApoE 4/4 displayed the highest influence on TG a median of 2.94mmol/L (IQR 1.75 – 5.13 mmol/L) as well as the highest non-HDL with a median of 4.68 mmol/L (IQR3.64 – 5.55 mmol/L). The carriers of the homozygous ApoE 4/4 with the best glycaemic control had two of the highest lipoproteins, TG and non-HDL. These results are interesting in that carrier of ApoE genotypes showing poor glycaemic control do not display dyslipidaemia as expected in secondary dyslipidaemia.

3.8 Comparisons of measured parameters between PCSK9 genotypes in dyslipidaemic participants

Similar to the ApoE genotype the effects or influence of PCSK9 genotype on each of the

measured analytes: HbA1c, TC, TG, LDL and HDL was assessed. However, unlike ApoE genotype, the comparison was only done for PCSK9 rs505151 genotypes because it has two well distributed variants, PCSK9 rs505151 A/A (55%) and PCSK9 rs505151 G/A (41%) and a small fraction of PCSK9 rs505151 A/A with only 4%. Similar comparative analysis was not possible for PCSK9 rs28362286 genotype because PCSK9 rs28362286 C/C constitutes 94%, PCSK9 rs28362286 C/A constituting 6% and PCSK9 rs28362286 A/A genotype being completely absent. The results of the PCSK9 genotypes influence on the measured parameters for the dyslipidaemic participants irrespective of the ApoE genotype genotypes are summarised in Figure 3.7 below. The correlation of the PCSK9 rs505151 genotypes and measured parameters did not show any significant difference across all the parameters. However, carriers of PCSK9 rs505151 A/A genotype had higher HbA1c with a median of 10.10% (IQR 7.48 – 12.9) compared to PCSK9 rs505151G/A genotype with a median of 9.00% (IQR 7.03 – 11.35).

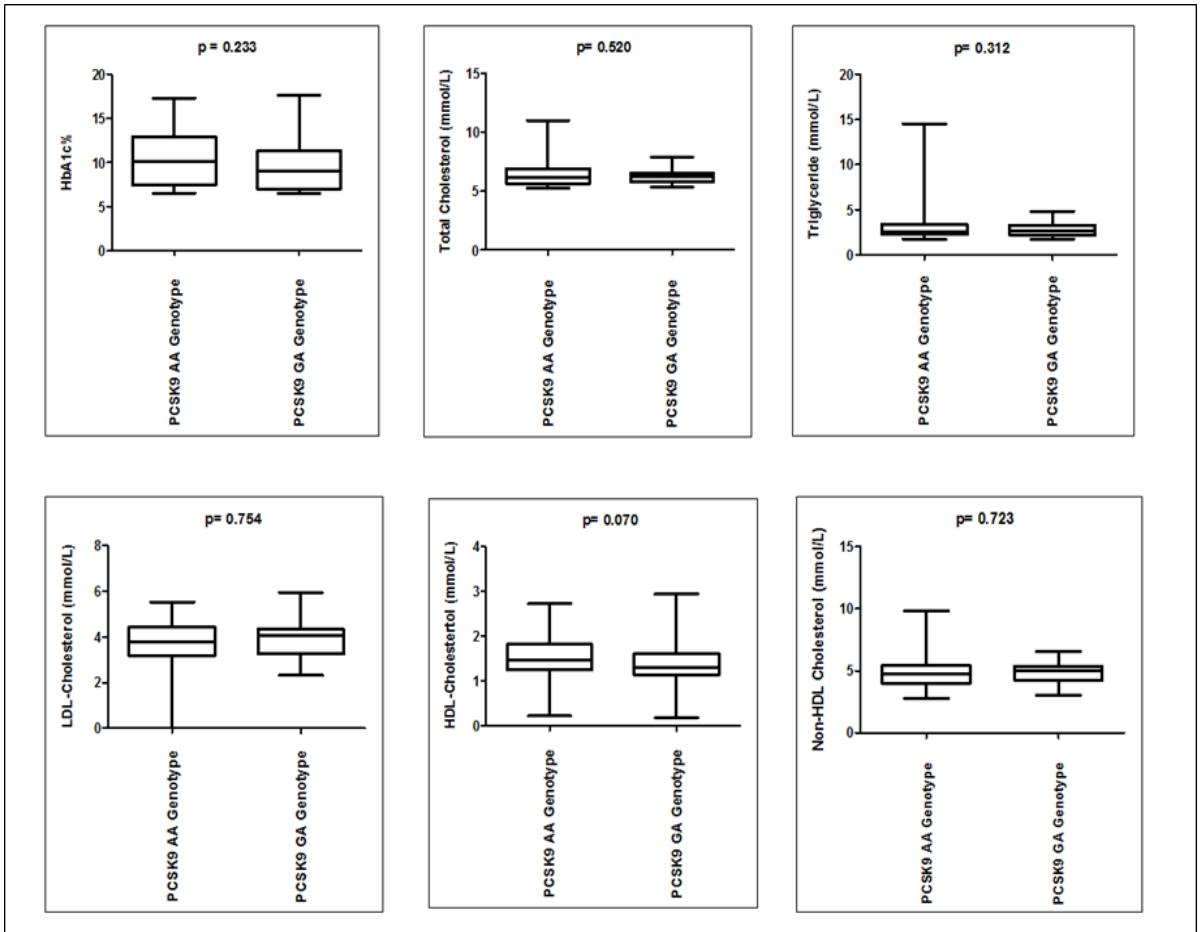


Figure 3.7 Influence of PCSK9 genotypes on the measured parameters

The results show that even though carriers of PCSK9rs505151G/A had lower HbA1c, they had higher atherogenic associated characteristics; higher TC, LDL, TG and non-HDL as well as lower HDL compared to PCSK9rs505151A/A genotype.

3.9 Gene-gene (ApoE-PCSK9) interactions

The results shown in Table 3.6 below compare measured parameters between PCSK9 rs505151 G/A and PCSK9 rs505151 A/A in each specific ApoE genotype. Even though they did not reach statistical significance, PCSK9 rs505151 G/A had higher values in most cases than PCSK9 rs505151 A/A except for the ApoE X where PCSK9 rs505151A/A had higher levels. Noted in these results was that the PCSK9 rs505151 A/A- ApoE2/2 interaction had higher HbA1c but lower lipid results than other genetic interactions tested. An opposite picture is noted in the PCSK9rs505151G/A-ApoE X had higher HbA1c but lower lipid levels. Another observation was the significant difference between PCSK9 G/A-ApoE2/3 HDL compared to PCSK9 A/A-ApoE2/3 participants ($p= 0.02$). Considering that ApoE genotypes were similar for each pair that was being compared, the difference can be attributed to the different PCSK9 genotypes.

Table 3.10 Lipid comparison between PCSK9 rs505151 G/A and PCSK9 rs505151 A/A in each ApoE Genotype

Genotype		HbA1c	TC	TG	LDL	HDL	Non-HDL
PCSK9 G/A-ApoE2/2	Median (IQR)	5.72 (5.08-12.25)	5.41 (3.33-6.59)	2.32 (1.48-2.63)	2.74 (1.50-3.80	1.51 (1.03-1.82)	3.91 (2.19-4.88)
PCSK9 A/A-ApoE2/2	Median (IQR)	9.5 (5.80-14.30)	4.74 (3.20-6.45)	1.98 (1.40-2.47)	2.65 (1.40-3.89)	1.38 (0.93-1.76)	3.78 (1.81-5.07)
p- value		0.54	0.85	0.47	0.94	0.94	0.83
PCSK9 G/A-ApoE2/3	Median (IQR)	5.00 (4.80-6.30)	4.30 (3.62-5.05)	1.32 (0.77-1.79)	2.15 (1.85-3.09)	1.3 (1.12-1.57)	3.00 (2.33-3.70)
PCSK9 A/A-ApoE2/3	Median (IQR)	5.9 (5.25-8.80)	3.83 (3.12-4.95)	1.34 (0.71-1.98)	2.16 (1.81-2.85)	1.02 (0.76-1.33)	2.81 (2.23-3.77)
p- value		0.18	0.53	0.98	0.88	0.02	0.97
PCSK9 G/A-ApoE3/3	Median (IQR)	7.70 (5.23-9.08)	6.24 (5.10-6.54)	2.12 (1.48-3.15)	3.71 (2.70-4.34)	1.21 (0.95-1.31)	5.14 (3.69-5.40)
PCSK9 A/A-ApoE3/3	Median (IQR)	5.90 (5.50-7.55)	5.46 (4.26-6.21)	1.40 (0.97-2.81)	2.88 (2.17-3.87)	1.43 (1.07-1.78)	3.84 (2.87-4.89)
p- value		0.37	0.43	0.32	0.07	0.18	0.18
PCSK9 G/A-ApoE3/4	Median (IQR)	6.35 (5.10-9.23)	5.60 (4.92-6.29)	1.91 (1.11-3.08)	2.89 (1.79-4.14)	1.27 (1.01-1.47)	4.17 (3.69-4.91)
PCSK9 A/A-ApoE3/4	Median (IQR)	6.20 (5.48-10.15)	5.40 (3.87-6.44)	1.54 (1.13-2.37)	3.36 (2.50-4.20)	1.21 (0.94-1.49)	3.82 (2.89-5.19)
p- value		0.83	0.58	0.98	0.25	0.89	0.58
PCSK9 G/A-ApoE X	Median (IQR))	6.8 (4.10-8.70)	4.85 (4.02-5.96)	2.71 (1.49-3.38)	2.46 (2.09-3.44)	1.21 (0.70-1.48)	3.75 (3.20-4.55)
PCSK9 A/A-ApoE X	Median (IQR)	6.3 (5.25-13.60)	5.27 (4.62-6.19)	1.52 (1.06-2.55)	3.30 (2.53-4.20)	1.33 (0.77-1.64)	3.90 (3.45-4.74)
p- value		0.36	0.56	0.45	0.27	0.5	0.67

G/A -Proprotein convertase subtilisinlike/kexin type 9 - PCSK9rs505151G/A

PCSK9 A/A-Proprotein convertase subtilisinlike/kexin type 9-PCSK9rs505151A/A

HbA1c- Glycated haemoglobin; TC- Total Cholesterol; TG – Triglycerides: LDL-Low density lipoprotein

HDL- High density lipoprotein and Non-HDL – Non-High-density lipoprotein

The table primarily shows comparisons of measured parameters between the two PCSK9 genotypes and the different ApoE genotypes. Assessment of possible genetic interaction with the ApoE irrespective of PCSK9 genotype and PCSK9 genotype irrespective of the ApoE genotypes did not show any form of synergy nor redundancy.

CHAPTER 4: DISCUSSION

4.1. New findings

The aim of this study was to evaluate the role of ApoE and PCSK9 genetic variation on the type of dyslipidaemia that develops in South African diabetic patients. We hypothesized that different ApoE and PCSK9 variants influence the type or form of dyslipidaemia that develops in DM. The study established the presence and characterisation of dyslipidaemia in diabetic participants. It demonstrated that dyslipidaemia that develops in diabetes is not only due to poor glycaemic control but there are underlying genetic factors involved. The study produced the first report on the distribution of ApoE and PCSK9 between dyslipidaemic and non-dyslipidaemic diabetic participants. The findings, which are reported here for the first time, are that;

i) ApoE2/3 diabetics are less likely to develop dyslipidaemia of any form
ii) ii) ApoE3/3 tend to develop dyslipidaemia,
iii) PCSK9 rs505151 GA carriers are more likely to develop dyslipidaemia compared to PCSK9 rs505151 AA carriers.
iv) An incidental, possibly novel finding until verified was the presence of an uncharacterised ApoE which was labelled as X. The process of characterisation involves isoelectric focusing, comparison with ApoE3 binding ability to LDL (Apo B, E) receptors on cultured fibroblasts, which are beyond the scope of this project.

4.2 Measured analytes

The data presented shows that HbA1c is higher in older participants. This is similar to reports from other studies (Arnetz et al., 1982; Yang et al., 1997). However, the higher HbA1c might not be due to poor glycaemic control because others studies have demonstrated that aging is associated with increased HbA1c levels, independent of glucose levels and insulin resistance (Dubowitz et al., 2014). There are no studies on the effects of aging on HbA1c in Africa. Such a study would further interrogate the diagnostic specificity of HbA1c. Some researchers recommended HbA1c to be considered as a predictor of dyslipidaemia in T2DM in addition to its role as glycaemic control monitor (Singh and Kumar, 2011; Hussain et al.,2017). HbA1c was positively correlated with high triglyceride. Hence, the recommendation by other researchers for its use as an indicator of triglyceride level and predictor of cardiovascular risk factors in T2DM (Zaidi. et al., 2015; Naqvi et al., 2017). Correlation of lipoproteins showed a

significant correlation of LDL with several parameters that are implicated in cardiovascular pathology. The positive correlation with blood pressure has been reported by many researchers (Turner et al., 1998; Moro et al., 1999; Verges, 2005). This is due to the LDL increased plasma residence time in T2DM which promote cholesterol deposition in the arterial wall (Verges, 2005; Lyons and Jenkins, 1997). LDL correlation was similar in many instances to TC because it is the largest fraction of TC. HDL results were higher than those reported by other researchers (Persegol et al., 2006; Taskinen et al., 1984). In this study age positively correlated with HDL.

4.3 Apo E genotype frequency

Except for a few areas outlined below, the ApoE genotype and allele frequency in this study closely resembles those in some African countries like the Central African Republic, West, Central and East Africa (Zekraoui et al., 1997). Compared to a study by Masemola et al., (2007) that looked at ApoE genotypes and their relation to lipid levels in a rural South African population, the difference is that homozygous ApoE2/2 was absent (0%) in that study compared to 5.9% and 5% in dyslipidaemic and non dyslipidaemic diabetic participants respectively. Studies among the central, southern, west and east African population reported an ApoE2/2 frequency distribution of 2.3%, (Zekraoui et al., 1997). This constitutes 50% of what has been found in this study. Homozygous ApoE3/3 and ApoE4/4 had a similar distribution pattern with the above-mentioned studies. Results in this study found a frequency distribution of 20.6% among the heterozygous ApoE2/3 non dyslipidaemic participants. This was similar to 20.2% reported among the rural South African population (Masemola et al., 2007). An interesting significant ($p < 0.0001$) finding in this study is that dyslipidaemic participants had a distribution of only 5.3%, compared to 20.6% among the non dyslipidaemic participants. This finding the first of its kind, can be interpreted to mean that heterozygous ApoE2/3 diabetic patients seldomly develop dyslipidaemia. Another interesting discovery of this study is the frequency distribution of 1.8% among the heterozygous ApoE2/4 dyslipidaemic participants, but (0%) among the non dyslipidaemic participants. Zekraoui et al., (1997) reported a 4.3% frequency distribution in the African population mentioned above. Masemola et al., (2007) reported 17%, compared to its very low frequency among the dyslipidaemic participants; and absence in the non-dyslipidaemic diabetic participants, may be interpreted that heterozygous ApoE2/4 carriers seldomly develop DM.

In summary, the study showed for the first time that heterozygous ApoE2/3 DM seldomly develop dyslipidaemia while heterozygous ApoE2/4 seldomly develop DM. The similarities in the ApoE genotype frequencies distribution between the non dyslipidaemic participants in this study and those reported for the general black population in the North-West Province of South Africa (Loktionov et al., 1999) and the rural South African population in Limpopo of South Africa (Masemola et al., 2007) as well as Caucasians of western Europe (Lucotte et al., 1997), justifies the n number used in the study.

4.4 PCSK9 variant frequency distribution

This study evaluated the frequency distribution of PCSK9rs505151G>A and PCSK9rs28362286C>A in both the dyslipidaemic and non-dyslipidaemic participants. The study provides the first report on the distribution of PCSK9 rs505151C>A and PCSK9rs28362286C>A genotypes in diabetic patients in South Africa. PCSK9 rs505151AA was 55% in dyslipidaemic participants compared to 63% in the non-dyslipidaemic participants. PCSK9rs505151GA genotype had a notably higher frequency distribution in the dyslipidaemic (41%) compared to the non-dyslipidaemic (27%). This difference had tendency to significance ($p = 0.06$), which with a higher n number might be significant. However, this tendency to significance maybe interpreted to mean that PCSK9rs505151GA genotype has predisposition to development of dyslipidaemia in diabetic subjects. PCSK9rs505151GG genotype was two-fold more in non dyslipidaemic (10%) compared to dyslipidaemic (4%), therefore, similar to PCSK9rs505151AA genotype might be interpreted to infer protection from development of dyslipidaemia.

In a study undertaken to detect the association of PCSK9rs505151G>A with serum lipid levels in the Guangxi Bai Ku Yao and Han Chinese population reported a frequency distribution that is different from the results of the present study. The Guangxi Bai Ku Yao and Han populations had a frequency of 95.99%, 4.01% and 0% in Guangxi Bai Ku Yao, and 91.02%, 8.36% and 0.62% for A/A, A/G and G/G genotypes respectively (Aung et al., 2011) compared to 55%, 41% and 4% found in this study. The difference might be due to the fact that this study looked at selected (diabetic) population while the Chinese looked at a general population.

In this study, PCSK9 rs 28362286 A/A genotype was absent in both dyslipidaemic and non-dyslipidaemic participants. PCSK9 rs28362286 C/A is absent in the non dyslipidaemic participants with a very small percentage 5.8% in the dyslipidaemic participants. PCSK9 rs28362286 C/C genotype is predominant in both the dyslipidaemic (94.2%) and non-dyslipidaemic (100%). An investigation looking at predicting LDL levels in the black South African population, several PCSK9 variants including PCSK9 rs28362286 was examined and their frequency determined (van Zyl et al., 2014). They reported a frequency distribution of 1350/1428 (94.5%) for PCSK9 rs28362286 C/C and 78/1428 (5.5%) for PCSK9 rs28362286 C/A with 0% (absent) PCSK9 rs28362286 A/A, findings which are similar to results in this study. The absence of PCSK9 rs28362286 A/A in both dyslipidaemic and non dyslipidaemic as well as in the study by van Zyl et al., 2014, can mean that this genotype is absent in the black South African population. Absence of gene variants can be selective pressures on alleles that may underlie simple vs. complex disorders (Zwick et al., 2000).

4.5 ApoE genotypes influence on dyslipidaemia

The study demonstrated that those with poor glycaemic control did not show the dyslipidaemia as expected under the secondary dyslipidaemia classification. Even though only a few studies correlated glycaemic control to dyslipidaemia, the effects of ApoE4/4 in this study concurs with findings of exaggerated dyslipidaemia and atherosclerosis in diabetic mice with knock out LDLR and therefore supports the notion that ApoE4/4 could be central to diabetic dyslipidaemia (Johnson et al., 2011). The results are also consistent with studies in the Afro-Caribbean people where ApoE4/4 genotypes had higher TC levels compared with ApoE2/2 genotypes (Larifla et al., 2017). A study in Egypt that looked at ApoE gene variants as a risk factor for CAD in T2D patients found that ApoE4/4 had the highest TC (Halim et al., 2012). However, a large meta-analysis reported that carriers of the homozygous ApoE2/2 genotype had lower mean LDL levels than carriers of the homozygous ApoE4/4 genotype (Liu et al., 2014).

4.6 PCSK9 rs505151 genotypes influence on dyslipidaemia.

The study looked at the effects PCSK9 rs505151 genotypes on the levels of lipids and

lipoproteins. PCSK9 rs505151 A/A had lower but not significantly different LDL levels median 3.79mmol/L (IQR 3.17 – 4.42mmol/L) compared to PCSK9 rs505151 GA with LDL median of 4.08mmol/L (IQR 3.27 – 4.35mmol/L) p = 0.75. Even though there was no significant difference, the result an important finding because van Zyl et al., (2014) reported a significant difference in LDL levels between carriers of the homozygous PCSK9 rs28362286 C/C and carriers of heterozygous PCSK9 rs28362286 C/A p <0.0001. The Dallas Heart Study also identified three PCSK9 variants in the blacks and whites, which were associated with lower plasma levels of LDL. Therefore, these studies show that different PCSK9 variants have different effects on the lipoproteins (Kotowski et al., 2006). The absence of a direct reciprocal correlation between HbA1c levels as a marker of glycaemic control to lipoprotein levels as markers of dyslipidaemia, this small study seems to infer that development of cholesterol (LDL) related dyslipidaemia might be independent of the level of glycaemic control and therefore, not secondary to DM but that there may be another underlying factor, which may be genetic. This is the first report on the association of PCSK9, DM and dyslipidaemia.

4.7 Gene-gene interactions (genetic synergy or epistasis)

This forms a simplified discussion looking at genetic interaction to show if there is any possible form of synergy between two genes that are known to be associated with dyslipidaemia. There are no records on this type of analysis in the African population. Each of the ApoE genotype had a dual comparison between two PCSK9 genotypes. Out of all the ApoE genotypes, ApoE2/3 had interesting HDL results. CSK9rs505151G/A-ApoE2/3 had significantly higher (p = 0.02) HDL compared to PCSK9rs505151A/A-ApoE2/3. Since the ApoE was the same, the difference can be attributed to the different PCSK9 genotypes. Considering the association of high HDL and cardio protection, it can be interpreted that the combination of CSK9rs505151G/A-ApoE2/3 is more CSK9rs505151A/A-ApoE2/3 in DM. However, other combinations showed minor differences. For instance, subjects with the combination of PCSK9rs505151A/A and ApoE2/2 genotypes had the highest HbA1c, median of 9.5% (IQR 5.80 – 14.30). On the other hand, the combination of PCSK9rs505151G/A and ApoE2/3 genotypes had the lowest HbA1c median 5.0% (IQR 4.80 – 6.30). Subjects with the combination of PCSK9rs505151G/A and ApoE3/3 genotypes had the highest TC, median 7.70mmol/L (IQR 5.23 – 9.08 mmol/L) but with nearly 50% of HbA1c in PCSK9 rs505151A/A and ApoE2/2 subjects. The combination of PCSK9rs505151A/A and ApoE2/3

genotypes had the lowest total cholesterol median 3.83 mmol/L (IQR 3.12 – 4.95 mmol/L). Other studies that did not consider gene interaction reported that APOE ε4 alleles are considered a risk for hypercholesterolemia (Saunders et al., 1993). However, there is considerable disagreement regarding the degree of hypercholesterolemia between ApoE3/3 and ApoE4/4 (Knouff et al., 1999). As can be noted, this study agrees with ApoE3/3 being associated with hypercholesterolemia. Participants with the combination of PCSK9rs505151G/A and ApoE X had the highest TG median 2.71mmol/L (IQR 1.49 – 3.38 mmol/L) ApoE and PCSK9 effects on TG as well as ApoE X have not been reported before. Carriers with a combination of PCSK9rs505151G/A and ApoE3/3 genotypes had the highest Non-HDL median 5.14 mmol/L (IQR 3.69 – 5.40 mmol/L). Carriers of the PCSK9rs505151A/A and ApoE2/3 genotypes had the lowest HDL median 1.02 mmol/L (0.76-1.33 mmol/L). Several reports give a general relationship between ApoE and HDL without the possible ApoE isoform associations (El Harchaoui et al., 2009; Ćwiklińska et al., 2015).

The study results show that the levels of serum LDL were slightly higher in the subjects with PCSK9rs505151G/A genotype than those with PCSK9rs505151AA genotype; the difference did not reach statistical significance. Several other studies have reported low LDL in some PCSK9 variants. In the PROSPER Study, PCSK9 R46L SNP was reported to lower LDL without an effect on the risk CVD in the elderly population with a high prevalence of cardiovascular disease (Polisecki et al., 2008). Other studies have reported PCSK9 genetic variants that persistently lower serum LDL levels than non-carrier black men. The reduction in LDL levels is associated with reduced subclinical atherosclerosis burden (Huang et al., 2009). However, analysis of the effects of two genes ApoE and PCSK9 genotypes displayed different patterns of dyslipidaemia in DM and this might be one of the reasons behind the absence as well as the different types of dyslipidaemia encountered.

CHAPTER 5: STRENGHTS AND LIMITATIONS, CONCLUSION AND RECOMMENDATIONS

5.1 Strengths and limitations

This is the first study in South Africa that investigated the frequency and association of various genotypes in the ApoE and PCSK9 gene variants in DM. The study is the first to report on the distribution of ApoE and PCSK9 in the diabetic population. In addition to reporting on previously identified variants in the African and other populations, an uncharacterized variant referred to in this study as ApoE X was noted to have a strong association on development of dyslipidaemia. The absence of PCSK9rs28362286A/A genotype in the African population is confirmed. Even though there was no clinical significance between the different genotypes (ApoE and PCSK9) and measured parameters, the study showed that there was no direct reciprocal association between poor glycaemic control developments of dyslipidaemia. The inability to achieve statistical significance with some results showing a tendency to significance can be attributed to the small sample number. Even though the sample number was small, there are several results that are similar to those reported in larger studies.

5.2 Conclusion

The results revealed that there is no direct reciprocal relationship between glycaemic control and level or type of dyslipidaemia. Therefore, the study has clearly demonstrated that dyslipidaemia found in DM has an underlying genetic influence predisposition. In line with pharmacogenetics, application of standard management to diabetic patients with different ApoE genotypes will not yield optimum results. Many diabetologists across the world have at one stage or another discussed possible patient non-compliance resulting in poor glycaemic control leading to dyslipidaemia. The thought of possible underlying genetic causes has not received a lot of attention especially in the African patients. With these findings, consideration to explore possible underlying genetic predisposition is recommended especially in diabetic patients with dyslipidaemia that responds poorly to standard therapy.

5.3 Recommendations

With these findings, a recommendation to look at underlying genetic predisposition in diabetic patients presenting with dyslipidaemia is made. Other researchers of lipid metabolism (Ramasamy, 2016) have also made similar recommendations. Racial differences

in the response to statins have been reported in South Africa (Raal et al., 2013). Differences between Asians and Westerners have also been reported with recommendations to elucidate the gene factors (Naito et al., 2017). Lessons can be drawn from advances in diagnosis and management of infectious disease where culture and institution of treatment that used to take several weeks can now be done within hours due to advances in nanotechnology. Secondly, molecular studies from DNA extraction to genotyping that used to take several days can now be done within hours at costs much lower than in the past. Point of care testing for TC and TG is now already available. Development of dipstick with anti ApoE lipoproteins as a first step is a real possibility with finer details looking at the homozygous and heterozygous. What needs to be considered is that optimum management works out to be cost effective in the end.

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APPENDICES

Appendix Ai: University of Cape Town Ethics

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Human Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6626 • Facsimile [021] 406 6411
e-mail: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

12 February 2013

HREC REF: 089/2013

Prof C Dandara
Human Genetics
Level 3
Wernher and Beit North
IIDMM

Dear Prof Dandara

PROTOCOL TITLE: TYPE 2 DIABETES MELLITUS AND DYSLIPIDAEMIA: EFFECT OF GENETIC VARIATION IN THE AFRICAN POPULATION

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

Before the study can be approved:

1. Please submit the WITS HREC approval for the study and updated documents.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN ETHICS

S Thomas

Appendix Aii: University of the Witwatersrand Ethics

R14/49 Dr Donald M Tanyanyiwa

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130130

NAME: (Principal Investigator)	Dr Donald M Tanyanyiwa
DEPARTMENT:	Department of Chemical Pathology CH Baragwanath Academic Hospital
PROJECT TITLE:	Type 2 Diabetes Mellitus and Dyslipidaemia: Effect of Genetic Variation in the African Population
DATE CONSIDERED:	25/01/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Sindeep Bhana
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 04/03/2013

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 Secretary in Room 10004, 10th floor, Senate House, University.

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**.

Principal Investigator Signature Date 11th March 2013

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix Bi: Point of Care Glycated Haemoglobin (HbA1c) Procedure

Purpose

This document outlines policies and procedures pertaining to Hemoglobin A1C testing by DCA Vantage. In an effort to be concise, some information may be excluded from the manufacturer's procedure. It is recommended that operators familiarize themselves with the manufacturer's product information that accompanies each reagent kit and the operators' manual if one exists.

The DCA Vantage Analyzer is a semi-automated bench top system used for the quantitative determination of hemoglobin A_{1C} in blood. The measurement of hemoglobin A_{1C} concentration is recommended for monitoring the long-term care of persons with diabetes. This assay provides a convenient method for the *in vitro* measurement of percent concentration of hemoglobin A_{1c} in blood.

Test Principle

The DCA Vantage Analyzer uses a spectrophotometer to analyze the intensity of the light transmitted through the cartridge optical window. Whole blood is added to the reagent cartridge, the cartridge is inserted into the DCA Vantage Analyzer, and meaningful results are available in 6 minutes. All measurements and calculations are performed automatically by the DCA Analyzer, and the screen displays percent HbA_{1c} at the end of the assay. The following chemical reaction occurs within the cartridge: For the measurement of total hemoglobin, potassium ferricyanide is used to oxidize hemoglobin in the sample to methemoglobin. The methemoglobin then complexes with thiocyanate to form thiocyanmethemoglobin, the colored species that is measured. The extent of color development at 531 nm is proportional to the concentration of total hemoglobin in the sample. For the measurement of specific HbA_{1c}, an inhibition of latex agglutination assay is used. An agglutinator (synthetic polymer containing multiple copies of the immunoreactive portion of HbA_{1c}) causes agglutination of latex coated with HbA_{1c} specific mouse monoclonal antibody. This agglutination reaction causes increased scattering of light, which is measured as an increase in absorbance at 531 nm. HbA_{1c} in whole blood specimens competes for the limited number of antibody-latex binding sites causing an inhibition of agglutination and a
decreased scattering of light. The decreased scattering is measured as a decrease in absorbance at 531 nm. The HbA_{1c} concentration is then quantified using a calibration curve of absorbance versus HbA_{1c} concentration. The percent HbA_{1c} in the sample is then calculated as follows:

$\%\ HbA_{1c} = ([HbA_{1c}] / [\text{Total Hemoglobin}]) \times 100$

Both the concentration of hemoglobin A_{1c} specifically and the concentration of total hemoglobin are measured, and the ratio reported as percent hemoglobin A_{1c}.

Regulatory Requirements

I. Each testing site must have a documented quality control program, which is developed in collaboration with or has been approved by the MGH Pathology Service.

II. All test results must be maintained in patient records with all required information for four years
Required information:
1. Patient's name
2. Medical Record Number
3. Patient's gender
4. Patient's age or date of birth
5. Date & time test collected, performed and reported
6. Ordering Physician
7. Responsible physician (if not 6)
8. Reference or Target Range
9. Test Performed
10. Test units
11. Lab name

Test Procedure

Ensure that the system is in the **Home** screen, which displays the status of the system and is the starting point for Patient and Control Test Sequences. If the system is in the **Not Ready** state and you cannot initiate a Patient or Control Test Sequence, an alert message displays explaining why the system is not ready.

The **Recall menu** and the

System menu can also be accessed from the Home Screen.

1. Remove a reagent cartridge from the refrigerator and allow it to warm up to room temperature. (10 minutes in the unopened foil pouch, or 5 minutes if removed from the foil pouch.)

a. To open the foil pouch, tear down from the corner notch until the entire long side of the pouch is open. Do not use scissors. After opening the foil pouch, the reagent cartridge must be used within one hour.

b. When handling the reagent cartridge, do not touch or otherwise contaminate the optical window **(1)** or erroneous test results may occur.

c. Discard the reagent cartridge if any of the following conditions exist:

- The flexible pull-tab is loose or missing. **(2)**
- The desiccant bag is missing or open. **(3)**
- The cartridge is damaged.
- Loose desiccant particles are found inside the foil package.
- If the foil package is open for more than 60 minutes.

2. Collect the sample using the capillary holder.

3. Insert the capillary holder containing the sample into the reagent cartridge until the holder snaps into place. The open side of the capillary holder should face the foil pull tab.

4. Scan the Reagent Cartridge: Hold the reagent cartridge so that the barcode faces to the right. Insert the reagent cartridge above the "dot" located on the side of the instrument barcode track. Quickly and smoothly, slide the reagent cartridge down. A beep sounds to signal a successful scan.

5. With the barcode facing to the right, insert the reagent cartridge into the cartridge compartment until a gentle a snap is heard or felt. **NOTE:** The cartridge is designed to fit only one way into the system.

Do not force the cartridge into system.

6. Using a slow, continuous motion, pull the flexible pull-tab completely out of the reagent cartridge and discard.

7. Close the door. Five seconds after the door is closed, a beep sounds and the assay begins.

NOTE: If you accidentally close the door before you pull the flexible plastic tab, you have 5 seconds to re-open the door and pull the tab.

8. The Sample Data menu screen displays when the instrument detects the system door closes, and indicates a test is in progress after the 5-second delay.

9. Follow the prompts to enter patient's CSN (scan CSN from EPIC label on tube) and operator ID #.

10. The Result screen displays when the system finishes analyzing the sample. Press the "print" button on the screen to print results.

11. Remove the Reagent Cartridge

12. Open the cartridge compartment door.

13. Locate the button on the right side of the cartridge compartment.

14. Push and hold it down with your right hand.

15. With your left hand, gently push the tab on the cartridge to the right to release the cartridge.

16. Discard in biohazard container.

NOTE: You can cancel a test any time. To cancel a test, select Cancel. If a test in progress is cancelled, you must discard the sample.

Symbol Interpretation

result preceded by a less than (<) sign concentration is below the lower limit of the test (i.e. <2.5%)

result preceded by a greater than (>) sign Concentration is above the upper limit of the test (i.e. >14.0% for patient samples; >16.0% for control samples.) result followed by a minus sign (-) result is below the **Reference Range**

result followed by a plus sign (+) result is above the Reference range.

Reference ranges

Analytical Range

HgbA1C 3.8 – 6.4% None 3.0 – 10.0%

The tested ranges are those ranges that were tested during functional sensitivity and linearity testing.

***** Clinical policy and Procedure Manual

Laboratory Results: Guidelines for Retreving and Reporting.

Maintenance

Turn the power off and disconnect the power cord before cleaning (You can leave the DCA Vantage system on always, except during maintenance and cleaning procedures.)

Turning the system off

1. At the Home screen, select **Turn Off**. A message displays asking if you want to shut down the system.
2. Select **Yes**.
3. Turn the power switch to the off position when the system shut down is complete

Weekly

Cleaning:

Barcode Window: Clean the barcode window with a lint-free cloth dampened with water or ethanol

Exterior: Clean the exterior with a lint-free cloth dampened with water or ethanol. Do not allow liquid to drip into system.

NOTE: To disinfect the exterior of the system, expose the surface to 0.5% sodium hypochlorite for 10 minutes. Remove any visible blood on the system before disinfection.

Do not use any other type of solvent, oil, grease, or silicone spray on any part of the system

Running the Optical Test

See Operator's Manual for instructions

Document all instrument maintenance and cleaning on the appropriate log sheets

Connectivity and Downtime procedure

1. The DCA Vantage analyzer is connected to Telcor (POCT management system) through an interface on thenetwork.
2. In the event of connectivity failure, document the results on the patient's chart with the units and reference ranges. Results can be re-transmitted in the "Recall Results" menu.
3. Contact the POCT program for assistance. (ext.6-1462, 3-5392 or 6-3858)

Technical Assistance

Siemens Medical Solutions Technical Care Center: 1-877-229-3711

Customer Service: 1-800-255-3232

Serial Number:

Customer Account Number:

References

1. DCA Vantage ™ Analyzer Operator's Guide, Ref. 06489264, Rev. C, 2011-07.

Cross – References

DCA Vantage HgbA1C Training and Competency Assessment Record

DCA Vantage Maintenance Log

DCA Vantage Operator Training Checklist

DCA Vantage Optical Test Cartridge Results Log

Adapted from: POCT Program Massachusetts General Hospital - Pathology Service 55 Fruit Street, Boston, MA 02114

Appendix Bii: Central Laboratory Glycated Haemoglobin (HbA1c) Procedure

0104528123190COINV10.0

A1C-2

cobas®

Tina-quant Hemoglobin A1c Gen.2 Whole blood Application - Standardized according to IFCC transferable to DCCT/NGSP

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
04528123 190	Tina-quant Hemoglobin A1c Gen.2 (150 tests)	System-ID 07 6850 2	COBAS INTEGRA 400/400 plus COBAS INTEGRA 800
04528417 190	C.f.a.s. HbA1c (3 x 2 mL)	System-ID 07 6852 9	
05479207 190	PreciControl HbA1c norm (4 x 1 mL)	System-ID 07 7477 4	
05912504 190	PreciControl HbA1c path (4 x 1 mL)	System-ID 07 7478 2	
04528328 190	COBAS INTEGRA Hemolyzing reagent Gen.2 (6 x 10 mL)	System-ID 07 6851 0	

English

System information

Multitest A1CW2, test ID 0-028

Test HB-W2, test ID 0-128; test A1-W2, test ID 0-228

Ratio RWD2M, test ID 0-529 (acc. to DCCT/NGSP)

Ratio RWI2M, test ID 0-427 (acc. to IFCC)

Profile PA1W2, test ID 0-628

Intended use

In vitro test for the quantitative determination of mmol/mol hemoglobin A1c (IFCC) and % hemoglobin A1c (DCCT/NGSP) in whole blood on Roche clinical chemistry analyzers. HbA1c determinations are useful for monitoring of long-term blood glucose control in individuals with diabetes mellitus. Moreover, this test is to be used as an aid in diagnosis of diabetes and identifying patients who may be at risk for developing diabetes.

Summary[1,2,3,4,5,6,7,8,9]

Hemoglobin (Hb) consists of four protein subunits, each containing a heme moiety, and is the red-pigmented protein located in the erythrocytes. Its main function is to transport oxygen and carbon dioxide in blood. Each Hb molecule is able to bind four oxygen molecules. Hb consists of a variety of subfractions and derivatives. Among this heterogeneous group of hemoglobins HbA1c is one of the glycated hemoglobins, a subfraction formed by the attachment of various sugars to the Hb molecule. HbA1c is formed in two steps by the nonenzymatic reaction of glucose with the N-terminal amino group of the β-chain of normal adult Hb (HbA). The first step is reversible and yields labile HbA1c. This is rearranged to form stable HbA1c in a second reaction step.

In the erythrocytes, the relative amount of HbA converted to stable HbA1c increases with the average concentration of glucose in the blood. The conversion to stable HbA1c is limited by the erythrocyte's life span of approximately 100 to 120 days. As a result, HbA1c reflects the average blood glucose level during the preceding 2 to 3 months. HbA1c is thus suitable to monitor long-term blood glucose control in individuals with diabetes mellitus. Glucose levels closer to the time of the assay have a greater influence on the HbA1c level.[1]

The approximate relationship between HbA1c and mean blood glucose values during the preceding 2 to 3 months was analyzed in several studies. A recent study obtained the following correlation:

IFCC standardization (recalculated acc. to ref. 8)

- Estimated average glucose [mmol/L] = 0.146 x HbA1c (mmol/mol) + 0.834
  or
- Estimated average glucose [mg/dL] = 2.64 x HbA1c (mmol/mol) + 15.03

Standardization acc. to DCCT/NGSP[8]

- Estimated average glucose [mmol/L] = 1.59 x HbA1c (%) - 2.59
  or
- Estimated average glucose [mg/dL] = 28.7 x HbA1c (%) - 46.7

The risk of diabetic complications, such as diabetic nephropathy and retinopathy, increases with poor metabolic control. In accordance with its function as an indicator for the mean blood glucose level, HbA1c predicts the development of diabetic complications in diabetes patients.[4,5]

For monitoring of long term glycemic control, testing every 3 to 4 months is generally sufficient. In certain clinical situations, such as gestational diabetes, or after a major change in therapy, it may be useful to measure HbA1c in 2 to 4 week intervals.[7]

Test principle[10,11,12]

The anticoagulated whole blood specimen is hemolyzed automatically on the COBAS INTEGRA 400/400 plus/800 analyzers with COBAS INTEGRA Hemolyzing Reagent Gen.2. This method uses TTAB[a)] as the detergent in the hemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes). Sample pretreatment to remove labile HbA1c is not necessary.

All hemoglobin variants which are glycated at the β-chain N-terminus and which have antibody-recognizable regions identical to that of HbA1c are determined by this assay. Consequently, the metabolic state of diabetic patients having uremia or the most frequent hemoglobinopathies (HbAS, HbAC, HbAE) can be determined by this assay.[13,14]

Hemoglobin A1c

The HbA1c determination is based on the turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood.

- Sample and addition of R1 (buffer/antibody):
  Glycohemoglobin (HbA1c) in the sample reacts with anti-HbA1c antibody to form soluble antigen-antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, complex formation does not take place.
- Addition of SR (buffer/polyhapten) and start of reaction:
  The polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be determined turbidimetrically.

Hemoglobin

Liberated hemoglobin in the hemolyzed sample is converted to a derivative having a characteristic absorption spectrum which is measured bichromatically during the preincubation phase (sample + R1) of the above immunological reaction. A separate Hb reagent is consequently not necessary.

The final result is expressed as mmol/mol HbA1c or % HbA1c and is calculated from the HbA1c/Hb ratio as follows:

Protocol 1 (mmol/mol HbA1c acc. to IFCC):

HbA1c (mmol/mol) = (HbA1c/Hb) x 1000

Protocol 2 (% HbA1c acc. to DCCT/NGSP):

HbA1c (%) = (HbA1c/Hb) x 91.5 + 2.15

a) TTAB = Tetradecyltrimethylammonium bromide

A1C-2

cobas®

Tina-quant Hemoglobin A1c Gen.2 Whole blood Application - Standardized according to IFCC transferable to DCCT/NGSP

Reagents - working solutions

R1	Antibody reagent MES[b] buffer: 0.025 mol/L; TRIS[c] buffer: 0.015 mol/L, pH 6.2; HbA1c antibody (ovine serum): ≥ 0.5 mg/mL; stabilizers; preservatives
SR	Polyhapten reagent MES buffer: 0.025 mol/L; TRIS buffer: 0.015 mol/L, pH 6.2; HbA1c polyhapten: ≥ 8 µg/mL; stabilizers; preservatives

b) MES = 2-morpholinoethane sulfonic acid

c) TRIS = Tris(hydroxymethyl)-aminomethane

R1 is in position B and SR is in position C.

Precautions and warnings

Pay attention to all precautions and warnings listed in Section 1 / Introduction of this Method Manual.

Reagent handling

Ready for use

Storage and stability

Reagent

Shelf life at 2-8 °C	See expiration date on **cobas c** pack label
COBAS INTEGRA 400/400 plus systems	
On-board in use at 10-15 °C	4 weeks
COBAS INTEGRA 800 system	
On-board in use at 8 °C	4 weeks

Hemolyzing reagent

Shelf life at 2-8 °C	See expiration date on bottle label
COBAS INTEGRA 400/400 plus/800 systems	
On-board in use, ISE rack, closed bottles	4 weeks
On-board in use, multi rack, open bottles	2 days
COBAS INTEGRA 800 CTS system	
On-board in use, ISE rack, open bottles	2 days

When storing at temperatures under 3 °C, the reagent may become cloudy. This has no effect on the function of the reagent and is reversible at higher temperatures. It is therefore recommended to equilibrate the reagent at room temperature for approximately 10 minutes and mix thoroughly before use.

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable. Anticoagulated venous or capillary blood. The only acceptable anticoagulants are Li-heparin, K_2-EDTA, K_3-EDTA and potassium fluoride/Na_2-EDTA.

Stability:[15]	3 days at 15-25 °C
	7 days at 2-8 °C
	6 months at (-15)-(-25) °C

The recovery of HbA1c ratio values from sedimented samples, especially in case of poorly controlled diabetic patients, may be slightly elevated. To minimize this effect samples may be gently mixed by inversion prior to analysis.

Freeze only once. Mix specimen thoroughly after thawing.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

COBAS INTEGRA Hemolyzing Reagent Gen.2 for Tina-quant HbA1C Gen.2, Cat. No. 04528328 190, system-ID 07 6851 0.

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

Applications for Hb and HbA1c

COBAS INTEGRA 400/400 plus test definition Hb

Abbreviated test name	HB-W2
Measuring mode	Absorbance
Abs. calculation mode	Endpoint
Reaction mode	R1-S
Reaction direction	Increase
Wavelength A/B	378/659 nm
Calc. first/last	17/33
Predilution factor	100
Unit	g/dL

Pipetting parameters

Hb		Diluent (H_2O)
R1	120 µL	
Sample	6 µL	0 µL
Total volume	126 µL	

COBAS INTEGRA 400/400 plus test definition HbA1c

Abbreviated test name	A1-W2
Measuring mode	Absorbance
Abs. calculation mode	Endpoint
Reaction mode	R1-S-SR
Reaction direction	Increase
Wavelength A/B	340/659 nm
Calc. first/last	33/57
Predilution factor	100
Unit	g/dL

Pipetting parameters

HbA1c		Diluent (H_2O)
R1	120 µL	
Sample	6 µL	0 µL
SR	24 µL	0 µL
Total volume	150 µL	

COBAS INTEGRA 800 test definition Hb

Abbreviated test name	HB-W2
Measuring mode	Absorbance
Abs. calculation mode	Endpoint
Reaction mode	R1-S
Reaction direction	Increase

A1C-2

cobas®

Tina-quant Hemoglobin A1c Gen.2 Whole blood Application - Standardized according to IFCC transferable to DCCT/NGSP

Wavelength A/B	378/659 nm
Calc. first/last	17/44
Predilution factor	100
Unit	g/dL

Pipetting parameters

Hb		Diluent (H_2O)
R1	120 µL	
Sample	6 µL	0 µL
Total volume	126 µL	

COBAS INTEGRA 800 test definition HbA1c

Abbreviated test name	A1-W2
Measuring mode	Absorbance
Abs. calculation mode	Endpoint
Reaction mode	R1-S-SR
Reaction direction	Increase
Wavelength A/B	340/659 nm
Calc. first/last	44/96
Predilution factor	100
Unit	g/dL

Pipetting parameters

HbA1c		Diluent (H_2O)
R1	120 µL	
Sample	6 µL	0 µL
SR	24 µL	0 µL
Total volume	150 µL	

Ratio definition for mmol/mol HbA1c and % HbA1c calculation

Protocol 1 (mmol/mol HbA1c acc. to IFCC):

Abbreviated ratio name	**RWI2M (0-427)**
Equation	(A1-W2/HB-W2) x 1000
Unit	mM/M

Protocol 2 (% HbA1c acc. to DCCT/NGSP):

Abbreviated ratio name	**RWD2M (0-529)**
Equation	(A1-W2/HB-W2) x 91.5 + 2.15
Unit	%

Use the predefined profile (PA1W2, 0-628) for simultaneous order entry of Hb (HB-W2) and HbA1c (A1-W2) tests from the same hemolysate.

The ratio for HbA1c (mmol/mol HbA1c acc. to IFCC and % HbA1c acc. to DCCT/NGSP) will be automatically calculated after result output of both tests.

For dual reporting of both mmol/mol HbA1c (IFCC) units as well as % HbA1c (DCCT/NGSP) units please ensure that both ratio tests 0-427 (acc. to IFCC) and 0-529 (acc. to DCCT/NGSP) are activated.

Calibration

Hb

Calibrator	C.f.a.s. HbA1c

HbA1c

Calibrator	C.f.a.s. HbA1c
Calibration dilution ratio	1:1, 1:1.67, 1:2, 1:5, 1:25, 0, performed automatically by the instrument
Calibrator diluent	COBAS INTEGRA Hemolyzing Reagent Gen.2, Cat. No. 04528328 190
Calibration mode	Logit/log 5
Calibration replicate	Duplicate recommended
Calibration interval	Each lot, every 29 days, and as required following quality control procedures

Traceability: This method has been standardized against the approved IFCC reference method for the measurement of HbA1c in human blood[16,17] and can be transferred to results traceable to DCCT/NGSP by calculation.

Note

For these applications C.f.a.s. HbA1c calibrator values are reagent lot matched. For each application and each combination of C.f.a.s. HbA1c calibrator lot and Tina-quant HbA1c Gen.2 reagent lot the exact calibrator values are given in the respective value sheet for C.f.a.s. HbA1c calibrator. Enter the assigned lot-specific and application-specific value of the calibrator. Use the appropriate C.f.a.s. HbA1c calibrator only.
COBAS INTEGRA Hemolyzing Reagent Gen.2, 6 x 11 mL, Cat. No. 04528328 190, system-ID 07 6851 0, needs to be available on the analyzer. Otherwise the calibration cannot be carried out.

Quality control

Quality control	PreciControl HbA1c norm
	PreciControl HbA1c path
Control interval	24 hours recommended
Control sequence	User defined
Control after calibration	Recommended

For quality control, use control materials as listed in the "Order information" section. In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Note

Pretreat controls in the same way as samples.
HbA1c controls carry a declaration for mmol/mol HbA1c (IFCC) and % HbA1c (DCCT/NGSP) only. No declarations for Hb and HbA1c concentrations are provided. As a consequence, HbA1c controls are handled like samples and cannot be included in the COBAS INTEGRA systems Quality Control Program.

Calculation

Hb

COBAS INTEGRA systems automatically calculate the Hb concentration of each sample. For more details, please refer to Data Analysis in the Online Help (COBAS INTEGRA 400/400 plus/800 analyzers).

HbA1c

COBAS INTEGRA systems automatically calculate the HbA1c concentration of each sample. For more details, please refer to Data Analysis in the Online Help
(COBAS INTEGRA 400/400 plus/800 analyzers).

HbA1c ratio calculation

For calculation of the mmol/mol HbA1c value (IFCC) and the % HbA1c value (DCCT/NGSP), refer to the **Test principle** and **Ratio definition for**

mmol/mol HbA1c and % HbA1c calculation sections in this method sheet.

Limitations - interference[13,14,18,19,20,21,22]

1. For diagnostic purposes, mmol/mol HbA1c values (IFCC) and % HbA1c values (DCCT/NGSP) should be used in conjunction with information from other diagnostic procedures and clinical evaluations.
2. The test is designed only for accurate and precise measurement of mmol/mol HbA1c (IFCC) and % HbA1c (DCCT/NGSP). The individual results for total Hb and HbA1c concentration should not be reported.
3. As a matter of principle, care must be taken when interpreting any HbA1c result from patients with Hb variants. Abnormal hemoglobins might affect the half life of the red cells or the in vivo glycation rates. In these cases even analytically correct results do not reflect the same level of glycemic control that would be expected in patients with normal hemoglobin.[23]
   Whenever it is suspected that the presence of an Hb variant (e.g. HbSS, HbCC, or HbSC) affects the correlation between the HbA1c value and glycemic control HbA1c must not be used for the diagnosis of diabetes mellitus.
4. Any cause of shortened erythrocyte survival or decrease in mean erythrocyte age will reduce exposure of erythrocytes to glucose with a consequent decrease in mmol/mol HbA1c values (IFCC) and % HbA1c values (DCCT/NGSP), even though the time-averaged blood glucose level may be elevated. Causes of shortened erythrocyte lifetime might be hemolytic anemia or other hemolytic diseases, homozygous sickle cell trait, pregnancy, recent significant or chronic blood loss, etc. Similarly, recent blood transfusions can alter the mmol/mol HbA1c values (IFCC) and % HbA1c values (DCCT/NGSP). Caution should be used when interpreting the HbA1c results from patients with these conditions. HbA1c must not be used for the diagnosis of diabetes mellitus in the presence of such conditions.
5. Glycated HbF is not detected as it does not contain the glycated β-chain that characterizes HbA1c. However, HbF is measured in the Total Hb assay and as a consequence, specimens containing high amounts of HbF (> 10 %) may result in lower than expected mmol/mol HbA1c values (IFCC) and % HbA1c values (DCCT/NGSP).[13,22]
6. mmol/mol HbA1c values (IFCC) and % HbA1c values (DCCT/NGSP) are not suitable for diagnosis of gestational diabetes.[24]
7. In very rare cases of rapidly evolving type 1 diabetes the increase of HbA1c values might be delayed compared to the acute increase in glucose concentrations. In these conditions diabetes mellitus must be diagnosed based on plasma glucose concentrations and/or the typical clinical symptoms.[24]

Criterion: Recovery within ± 10 % of initial value.

Icterus: No significant interference up to a conjugated and unconjugated bilirubin concentration of 1000 µmol/L or 60 mg/dL.

Lipemia (Intralipid):
COBAS NTEGRA 800 analyzer: No significant interference up to an Intralipid concentration of 800 mg/dL.
COBAS NTEGRA 400/400 plus analyzers: No significant interference up to an Intralipid concentration of 600 mg/dL.
There is poor correlation between the triglycerides concentration and turbidity.

Glycemia: No significant interference up to a glucose concentration of 55.5 mmol/L or 1000 mg/dL. A fasting sample is not required.

Rheumatoid factors: No significant interference up to a rheumatoid factor concentration of 750 IU/mL.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[25]

Other: No cross reactions with HbA0, HbA1a, HbA1b, acetylated hemoglobin, carbamylated hemoglobin, glycated albumin and labile HbA1c were found for the anti-HbA1c antibodies used in this kit.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special wash programming: The use of special wash steps is mandatory when certain test combinations are run together on COBAS INTEGRA analyzers. Refer to the Method Manual, Introduction, Extra Wash Cycles for further instructions.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

COBAS INTEGRA 400/400 plus analyzers:
Hb: 4-35 g/dL
HbA1c: 0.3-2.6 g/dL*

*The measuring range for HbA1c lies between 0.3 g/dL and the concentration of the highest standard. The test range stated above is based on a typical calibrator value of 2.6 g/dL.

This corresponds to a measuring range of 23-197 mmol/mol HbA1c (IFCC) and 4.3-19.5 % HbA1c (DCCT/NGSP) at a typical hemoglobin concentration of 13.2 g/dL.

COBAS INTEGRA 800 analyzer:
Hb: 4-35 g/dL
HbA1c: 0.3-3.4 g/dL

This corresponds to a measuring range of 23-258 mmol/mol HbA1c (IFCC) and 4.3-24.8 % HbA1c (DCCT/NGSP) at a typical hemoglobin concentration of 13.2 g/dL.

In rare cases of ">test rng" flags which might occur with the whole blood application remix the whole blood sample and repeat the analysis with the same settings.

Lower limits of measurement

Lower detection limit of the test:
Hb: 0.5 g/dL
HbA1c: 0.1 g/dL

A typical lower detection limit for the HbA1c ratio may be calculated, based on a given Hb concentration. Assuming a typical Hb concentration of 13.2 g/dL, the lower detection limit is 8 mmol/mol HbA1c (IFCC) or 2.9 % HbA1c (DCCT/NGSP).

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values

Protocol 1 (acc. to IFCC): 29-42 mmol/mol HbA1c[26]

Protocol 2 (acc. to DCCT/NGSP): 4.8-5.9 % HbA1c[26]

This reference range was obtained by measuring 474 well-characterized healthy individuals without diabetes mellitus. HbA1c levels higher than the upper end of this reference range are an indication of hyperglycemia during the preceding 2 to 3 months or longer. According to the recommendations of the American Diabetes Association values above 48 mmol/mol HbA1c (IFCC) or 6.5 % HbA1c (DCCT/NGSP) are suitable for the diagnosis of diabetes mellitus.[24,27] Patients with HbA1c values in the range of 39-46 mmol/mol HbA1c (IFCC) or 5.7-6.4 % HbA1c (DCCT/NGSP) may be at a risk of developing diabetes.[24,27]

HbA1c levels may reach 195 mmol/mol (IFCC) or 20 % (DCCT/NGSP) or higher in poorly controlled diabetes. Therapeutic action is suggested at levels above 64 mmol/mol HbA1c (IFCC) or 8 % HbA1c (DCCT/NGSP). Diabetes patients with HbA1c levels below 53 mmol/mol HbA1c (IFCC) or

7 % HbA1c (DCCT/NGSP) meet the goal of the American Diabetes Association.[19,28]

HbA1c levels below the established reference range may indicate recent episodes of hypoglycemia, the presence of Hb variants, or shortened lifetime of erythrocytes.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the COBAS INTEGRA analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (1 aliquot per run, 1 run per day, 21 days). The following results were obtained (data based on DCCT/NGSP values):

	Level 1		Level 2	
	Mean % HbA1c	CV %	Mean % HbA1c	CV %
Repeatability	5.4	0.8	10.2	0.9
Intermediate precision	5.3	1.3	10.3	1.0

Method comparison

% HbA1c (DCCT/NGSP) values for human blood samples obtained on a COBAS INTEGRA 400 analyzer using the COBAS INTEGRA Tina-quant Hemoglobin A1c Gen.2 reagent system (y) were compared with those determined using the COBAS INTEGRA Tina-quant Hemoglobin A1c Gen.2 reagent system (whole blood application) on a COBAS INTEGRA 800 analyzer (x). Samples were measured individually.

COBAS INTEGRA 400 analyzer	Sample size (n) = 69
Passing/Bablok[29]	Linear regression
y = 0.963x + 0.222 %	y = 0.961x + 0.267 %
τ = 0.963	r = 0.998
SD (md 95) = 0.201	Sy.x = 0.100

Values ranged from 4.91 to 12.5 % HbA1c (DCCT/NGSP).

% HbA1c (DCCT/NGSP) values for human blood samples obtained on a COBAS INTEGRA 800 analyzer using the COBAS INTEGRA Tina-quant Hemoglobin A1c Gen.2 reagent system (y) were compared with those determined using the COBAS INTEGRA Hemoglobin A1c reagent (whole blood application) on a COBAS INTEGRA 800 analyzer (x). Samples were measured individually.

COBAS INTEGRA 800 analyzer	Sample size (n) = 100
Passing/Bablok[29]	Linear regression
y = 0.970x + 0.263 %	y = 0.975x + 0.232 %
τ = 0.949	r = 0.995
SD (md 95) = 0.283	Sy.x = 0.138

Values ranged from 5.0 to 12.7 % HbA1c (DCCT/NGSP).

% HbA1c (DCCT/NGSP) values for human blood samples obtained on a COBAS INTEGRA 800 CTS analyzer using the COBAS INTEGRA Tina-quant Hemoglobin A1c Gen.2 reagent system (y) were compared with those determined on a COBAS INTEGRA 800 analyzer (whole blood application) (x). Samples were measured individually.

COBAS INTEGRA 800 CTS analyzer	Sample size (n) = 71
Passing/Bablok[29]	Linear regression
y = 0.987x + 0.064 %	y = 1.003x + 0.052 %
τ = 0.924	r = 0.997
SD (md 95) = 0.230	Sy.x = 0.106

Values ranged from 5.06 to 12.6 % HbA1c (DCCT/NGSP).

In addition, a comparison to a commercially available HPLC method was performed. The HPLC method was standardized in conformance with DCCT (Diabetes Control and Complications Trial).[3,4]

HPLC method	Sample size (n) = 60
Passing/Bablok[29]	Linear regression
y = 0.943x + 0.517 %	y = 0.932x + 0.572 %
τ = 0.957	r = 0.997
SD (md 95) = 0.235	Sy.x = 0.110

Values ranged from 4.9 to 12.1 % HbA1c (DCCT/NGSP).

Analytical specificity

Hb derivatives	Labile HbA1c (pre-HbA1c), acetylated Hb, and carbamylated Hb do not affect the assay result.
Hb variants	Specimens containing high amounts of HbF (> 10 %) may yield lower than expected HbA1c results.

Please note:

According to the consensus statement of the American Diabetes Association (ADA), the European Association for the Study of Diabetes (EASD), the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the International Diabetes Federation (IDF) HbA1c results should be reported in parallel, both in mmol/mol HbA1c (IFCC) and % HbA1c (DCCT/NGSP) values.[30] In addition an HbA1c derived estimated average glucose concentration can be reported which can be calculated according to the equations given in the summary section of this method sheet. Former % HbA1c (IFCC) values must not be used due to the risk of mix up / misinterpretation with the % HbA1c (DCCT/NGSP) values.

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A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
→	Volume after reconstitution or mixing

CE

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com

Roche

Appendix C: Total Cholesterol

00051685038190c701V4.0

CHOL2

cobas®

Cholesterol Gen.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
05168538 190	Cholesterol Gen.2 2100 tests	System-ID 05 6726 3	Roche/Hitachi **cobas c** 701/702
10759350 190	Calibrator f.a.s. (12 x 3 mL)	Code 401	
10759350 360	Calibrator f.a.s. (12 x 3 mL, for USA)	Code 401	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
10171735 122	Precinorm U (4 x 5 mL)	Code 300	
12149435 122	Precinorm U plus (10 x 3 mL)	Code 300	
12149435 160	Precinorm U plus (10 x 3 mL, for USA)	Code 300	
10781827 122	Precinorm L (4 x 3 mL)	Code 304	
10171778 122	Precipath U (20 x 5 mL)	Code 301	
10171760 122	Precipath U (4 x 5 mL)	Code 301	
12149443 122	Precipath U plus (10 x 3 mL)	Code 301	
12149443 160	Precipath U plus (10 x 3 mL, for USA)	Code 301	
11285874 122	Precipath L (4 x 3 mL)	Code 305	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
05172152 190	Diluent NaCl 9 % (119 mL)	System-ID 08 6869 3	

English

System information

CHO2I: ACN 8798: ID/MS Standardization

CHO2A: ACN 8433: Abell/Kendall Standardization

Intended use

In vitro test for the quantitative determination of cholesterol in human serum and plasma on Roche/Hitachi **cobas c** systems.

Summary

Cholesterol is a steroid with a secondary hydroxyl group in the C3 position. It is synthesized in many types of tissue, but particularly in the liver and intestinal wall. Approximately three quarters of cholesterol is newly synthesized and a quarter originates from dietary intake. Cholesterol assays are used for screening for atherosclerotic risk and in the diagnosis and treatment of disorders involving elevated cholesterol levels as well as lipid and lipoprotein metabolic disorders.

Cholesterol analysis was first reported by Liebermann in 1885 followed by Burchard in 1889. In the Liebermann-Burchard reaction, cholesterol forms a blue-green dye from polymeric unsaturated carbohydrates in an acetic acid/acetic anhydride/concentrated sulfuric acid medium. The Abell and Kendall method is specific for cholesterol, but is technically complex and requires the use of corrosive reagents. In 1974, Roeschlau and Allain described the first fully enzymatic method. This method is based on the determination of Δ4-cholestenone after enzymatic cleavage of the cholesterol ester by cholesterol esterase, conversion of cholesterol by cholesterol oxidase, and subsequent measurement by the Trinder reaction of the hydrogen peroxide formed. Optimization of ester cleavage (> 99.5 %) allows standardization using primary and secondary standards and a direct comparison with the CDC and NIST reference methods.[1,2,3,4,5,6,7,8,9] Nonfasting sample results may be slightly lower than fasting results.[10,11,12]

The Roche cholesterol assay meets the 1992 National Institutes of Health (NIH) goal of less than or equal to 3 % for both precision and bias.[12]

The assay is optionally standardized against Abell/Kendall and isotope dilution/mass spectrometry. The performance claims and data presented here are independent of the standardization.

Test principle

Enzymatic, colorimetric method.

Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide formed effects the oxidative coupling of phenol and 4-aminophenazone to form a red quinone-imine dye.

$$\text{Cholesterol esters} + H_2O \xrightarrow{CE} \text{cholesterol} + \text{RCOOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CHOD} \text{cholest-4-en-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AAP} + \text{phenol} \xrightarrow{POD} \text{quinone-imine dye} + 4\,H_2O$$

The color intensity of the dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance.

Reagents – working solutions

R1 PIPES buffer: 225 mmol/L, pH 6.8; Mg^{2+}: 10 mmol/L; sodium cholate: 0.6 mmol/L; 4-aminophenazone: ≥ 0.45 mmol/L; phenol: ≥ 12.6 mmol/L; fatty alcohol polyglycol ether: 3 %; cholesterol esterase (Pseudomonas spec.): ≥ 25 µkat/L (≥ 1.5 U/mL); cholesterol oxidase (E. coli): ≥ 7.5 µkat/L(≥ 0.45 U/mL); peroxidase (horseradish): ≥ 12.5 µkat/L (≥ 0.75 U/mL); stabilizers; preservative

R1 is in position B and C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

Reagent handling

Ready for use

Storage and stability

CHOL2

CHOL2

Cholesterol Gen.2

cobas®

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	4 weeks
On-board on the Reagent Manager:	24 hours

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	4 weeks
On-board on the Reagent Manager:	24 hours

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.
Serum.
Plasma: Li-heparin and K_2-EDTA plasma

Do not use citrate, oxalate or fluoride.[13]

Fasting and nonfasting samples can be used.[11]

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Stability:[14,15] 7 days at 15-25 °C
7 days at 2-8 °C
3 months at (-15)-(-25) °C

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section

General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma

cobas c 701/702 **test definition**

Assay type	1-Point		
Reaction time / Assay points	10 / 38		
Wavelength (sub/main)	700/505 nm		
Reaction direction	Increase		
Units	mmol/L (mg/dL, g/L)		
Reagent pipetting		Diluent (H_2O)	
R1	47 µL	93 µL	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	2 µL	15 µL	135 µL
Increased	4 µL	–	–

Calibration

Calibrators	S1: H_2O S2: C.f.a.s.
Calibration mode	Linear
Calibration frequency	Blank calibration - every 7 days - after reagent cassette change 2-point calibration - after reagent lot change - as required following quality control procedures

Traceability: This method has been standardized according to Abell/Kendall [12] and also by isotope dilution/mass spectrometry.[16]

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors: mmol/L x 38.66 = mg/dL
mmol/L x 0.3866 = g/L
mg/dL x 0.0259 = mmol/L

Limitations - interference

Criterion: Recovery within ± 10 % of initial values at a cholesterol concentration of 5.2 mmol/L (200 mg/dL).

Icterus:[17] No significant interference up to an I index of 16 for conjugated bilirubin and 14 for unconjugated bilirubin (approximate conjugated bilirubin concentration: 274 µmol/L or 16 mg/dL; approximate unconjugated bilirubin concentration: 239 µmol/L or 14 mg/dL).

Hemolysis:[17] No significant interference up to an H index of 700 (approximate hemoglobin concentration: 435 µmol/L or 700 mg/dL).

Lipemia (Intralipid):[17] No significant interference up to an L index of 2000. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[18,19]

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.[20]

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required. The latest version of the carry-over evasion list can also be found with the NaOHD/SMS/SmpCln1+2/SCCS Method Sheet and for further instructions refer to the operator's manual.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

CHOL2

Cholesterol Gen.2

cobas®

Limits and ranges

Measuring range

0.1-20.7 mmol/L (3.86-800 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:10 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 10.

Lower limits of measurement

Lower detection limit of the test

0.1 mmol/L (3.86 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Values below the lower detection limit (< 0.1 mmol/L) will not be flagged by the instrument.

Expected values

Clinical interpretation according to the recommendations of the European Atherosclerosis Society:[21]

	mmol/L	*mg/dL*	*Lipid metabolic disorder*
Cholesterol Triglycerides	< 5.2 < 2.3	(< 200) (< 200)	No
Cholesterol	5.2-7.8	(200-300)	Yes, if HDL-cholesterol < 0.9 mmol/L (< 35 mg/dL)
Cholesterol Triglycerides	> 7.8 > 2.3	(> 300) (> 200)	Yes

Recommendations of the NCEP Adult Treatment Panel for the following risk-cutoff thresholds for the US American population:[22]

Desirable cholesterol level	< 5.2 mmol/L	(< 200 mg/dL)
Borderline high cholesterol	5.2-6.2 mmol/L	(200-240 mg/dL)
High cholesterol	≥ 6.2 mmol/L	(≥ 240 mg/dL)

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

Repeatability	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Precinorm U	2.72 (105)	0.02 (1)	0.6
Precipath U	5.27 (204)	0.04 (2)	0.8
Human serum A	9.48 (367)	0.05 (2)	0.6
Human serum B	11.6 (449)	0.1 (4)	0.6
Human serum C	17.9 (692)	0.1 (4)	0.6
Intermediate precision	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Precinorm U	2.31 (89.3)	0.04 (1.6)	1.6
Precipath U	4.85 (188)	0.08 (3)	1.6
Human serum 3	1.97 (76.2)	0.03 (1.2)	1.6
Human serum 4	7.13 (276)	0.10 (4)	1.4

Results for intermediate precision were obtained on the master system **cobas c** 501 analyzer.

Method comparison

Cholesterol values for human serum and plasma samples obtained on a Roche/Hitachi **cobas c** 701 analyzer (y) were compared with those determined using the same reagent on a Roche/Hitachi **cobas c** 501 analyzer (x).

Sample size (n) = 84

Passing/Bablok[23]	Linear regression
y = 0.985x + 0.018 mmol/L	y = 0.986x + 0.009 mmol/L
τ = 0.985	r = 1.000

The sample concentrations were between 1.85 and 18.8 mmol/L (71.5 and 727 mg/dL).

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A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
→	Volume after reconstitution or mixing

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Roche Diagnostics warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label. THIS LIMITED WARRANTY IS IN LIEU OF ANY OTHER WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR PARTICULAR PURPOSE. IN NO EVENT SHALL ROCHE DIAGNOSTICS BE LIABLE FOR INCIDENTAL, INDIRECT, SPECIAL OR CONSEQUENTIAL DAMAGES.

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Appendix D: High Density Lipoprotein-cholesterol (HDL-C)

00043996031 90c501V8.0

HDLC3

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HDL-Cholesterol plus 3rd generation

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
04399803 190	HDL-Cholesterol plus 3rd generation 200 tests	System-ID 07 6833 2	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
12172623 122	Calibrator f.a.s. Lipids (3 x 1 mL)	Code 424	
12172623 160	Calibrator f.a.s. Lipids (3 x 1 mL, for USA)	Code 424	
10781827 122	Precinorm L (4 x 3 mL)	Code 304	
11778552 122	Precipath HDL/LDL-C (4 x 3 mL)	Code 319	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

HDLC3: ACN 435

For **cobas c** 502 analyzer:

HDLC3: ACN 8435

Intended use

In vitro diagnostic test for the quantitative determination of the HDL-cholesterol concentration in human serum and plasma on Roche/Hitachi **cobas c** systems.

Summary

High density lipoproteins (HDL) are responsible for the reverse transport of cholesterol from the peripheral cells to the liver. Here, cholesterol is transformed to bile acids which are excreted into the intestine via the biliary tract. Monitoring of HDL-cholesterol in serum is of clinical importance since an inverse correlation exists between serum HDL-cholesterol concentrations and the risk of atherosclerotic disease. Elevated HDL-cholesterol concentrations are protective against coronary heart disease, while reduced HDL-cholesterol concentrations, particularly in conjunction with elevated triglycerides, increase the cardiovascular risk.[1] Strategies have emerged to increase the level of HDL-cholesterol to treat cardiovascular disease.[2,3]

A variety of methods are available to determine HDL-cholesterol, including ultracentrifugation, electrophoresis, HPLC, precipitation-based methods and direct methods. Of these, the direct methods are used routinely. Several approaches for direct measurement of HDL-cholesterol in serum have been proposed, including the use of magnetically responsive particles as polyanion-metal combinations and the use of polyethylene glycol (PEG) with anti-apoprotein B and anti-apoprotein CIII antibodies.

This automated method for direct determination of HDL-cholesterol in serum and plasma uses PEG-modified enzymes and dextran sulfate. When cholesterol esterase and cholesterol oxidase enzymes are modified by PEG, they show selective catalytic activities toward lipoprotein fractions, with the reactivity increasing in the order:
LDL < VLDL ≈ chylomicrons < HDL.[4,5,6,7,8,9,10,11,12,13,14,15,16]

Non-fasting sample results are slightly lower than fasting results. Comparable non-fasting results were observed with the beta quantification method.[17,18,19]

The Roche direct HDL-cholesterol assay meets the 1998 National Institutes of Health (NIH) / National Cholesterol Education Program (NCEP) goals for acceptable performance.[20] The results of this method correlate with those obtained by precipitation-based methods and also by an ultracentrifugation method.

Test principle[4,5]

Homogeneous enzymatic colorimetric test.

In the presence of magnesium ions, dextran sulfate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG-modified enzymes.

The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx. 40 %).

Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase.

$$\text{HDL-cholesterol esters} + H_2O \xrightarrow{\text{PEG-cholesterol esterase}} \text{HDL-cholesterol} + \text{RCOOH}$$

In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ^4-cholestenone and hydrogen peroxide.

$$\text{HDL-cholesterol} + O_2 \xrightarrow{\text{PEG-cholesterol oxidase}} \Delta^4\text{-cholestenone} + H_2O_2$$

In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-amino-antipyrine and HSDA to form a purple-blue dye. The color intensity of this dye is directly proportional to the cholesterol concentration and is measured photometrically.

$$2\ H_2O_2 + \text{4-amino-antipyrine} + \text{HSDA}^* + H^+ \xrightarrow{\text{peroxidase}} \text{purple-blue pigment} + 5\ H_2O$$

*HSDA = Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline

Reagents - working solutions

R1 HEPES buffer: 10.07 mmol/L; CHES 96.95 mmol/L, pH 7.4; dextran sulfate: 1.5 g/L; magnesium nitrate hexahydrate: > 11.7 mmol/L; HSDA: 0.96 mmol/L; ascorbate oxidase (Eupenicillium sp., recombinant): > 50 µkat/L; peroxidase (horseradish): > 16.7 µkat/L; preservative

R2 HEPES buffer: 10.07 mmol/L, pH 7.0; PEG-cholesterol esterase (Pseudomonas spec.): > 3.33 µkat/L; PEG-cholesterol oxidase (Streptomyces sp., recombinant): > 127 µkat/L; peroxidase (horseradish): > 333 µkat/L; 4-amino-antipyrine: 2.46 mmol/L; preservative

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

Reagent handling

Ready for use

The intrinsic pink color of the cholesterol reagent does not interfere with the test.

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Storage and stability

HDLC3

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.
Serum.
Plasma: Li-heparin and K_2-EDTA plasma
EDTA plasma causes decreased results.[21] (See note in NCEP guideline section.)

Fasting and non-fasting samples can be used.[18] Collect blood by using an evacuated tube or syringe. Specimens should preferably be analyzed on the day of collection.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Stability:[19]	7 days at 2-8 °C
	30 days at (-60)-(-80) °C

It is reported that EDTA stabilizes lipoproteins.[22]

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section

General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma

cobas c 311 test definition

Assay type	2-Point End		
Reaction time / Assay points	10 / 6-33		
Wavelength (sub/main)	700/600 nm		
Reaction direction	Increase		
Units	mmol/L (mg/dL, g/L)		
Reagent pipetting		Diluent (H_2O)	
R1	150 µL	–	
R2	50 µL	–	

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2.5 µL	–	–
Decreased	12.5 µL	15 µL	135 µL
Increased	2.5 µL	–	–

cobas c 501 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 10-47
Wavelength (sub/main)	700/600 nm
Reaction direction	Increase
Units	mmol/L (mg/dL, g/L)
Reagent pipetting	Diluent (H_2O)
R1	150 µL –
R2	50 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2.5 µL	–	–
Decreased	12.5 µL	15 µL	135 µL
Increased	2.5 µL	–	–

cobas c 502 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 10-47
Wavelength (sub/main)	700/600 nm
Reaction direction	Increase
Units	mmol/L (mg/dL, g/L)
Reagent pipetting	Diluent (H_2O)
R1	150 µL –
R2	50 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2.5 µL	–	–
Decreased	12.5 µL	15 µL	135 µL
Increased	5.0 µL	–	–

Calibration

Calibrators	S1: H_2O S2: C.f.a.s. Lipids
Calibration mode	Linear
Calibration frequency	2-point calibration • after reagent lot change • as required following quality control procedures

Traceability:[19] This method has been standardized against the designated CDC reference method (designated comparison method).[20] The standardization meets the requirements of the "HDL Cholesterol Method Evaluation Protocol for Manufacturers" of the US National Reference System for Cholesterol, CRMLN (Cholesterol Reference Method Laboratory Network), November 1994.

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

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The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Quality control materials are intended for use only as monitors of accuracy and precision. The Laboratory Standardization Panel (LSP) of the National Cholesterol Education Program in the United States recommends two levels of controls, one in the normal range (0.9-1.7 mmol/L or 35-65 mg/dL) and one near the concentration for decision making (< 0.9 mmol/L or < 35 mg/dL).

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors: mmol/L x 38.66 = mg/dL
mmol/L x 0.3866 = g/L
mg/dL x 0.0259 = mmol/L

Limitations - interference[23]

Criterion: Recovery within ± 10 % of initial value at a HDL-cholesterol concentration of 1 mmol/L (38.7 mg/dL).

Icterus:[24] No significant interference up to an I index of 30 for conjugated and 60 for unconjugated bilirubin (approximate conjugated bilirubin concentration: 513 µmol/L or 30 mg/dL and approximate unconjugated bilirubin concentration: 1026 µmol/L or 60 mg/dL).

Hemolysis:[24] No significant interference up to an H index of 1200 (approximate hemoglobin concentration: 745 µmol/L or 1200 mg/dL).

Lipemia (Intralipid):[24] No significant interference up to an L index of 1800. No significant interference from native triglycerides up to 13.7 mmol/L or 1200 mg/dL. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Other: Elevated concentrations of free fatty acids and denatured proteins may cause falsely elevated HDL-cholesterol results.

In rare cases, elevated immunoglobulin concentrations can lead to artificially increased HDL-cholesterol results.

Ascorbic acid up to 2.84 mmol/L (50 mg/dL) does not interfere.

Abnormal liver function affects lipid metabolism; consequently, HDL and LDL results are of limited diagnostic value. In some patients with abnormal liver function, the HDL-cholesterol result may significantly differ from the DCM (designated comparison method) result.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[25,26]

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.[27]

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the carry-over evasion list can be found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1+2/SCCS Method Sheets. For further instructions refer to the operator's manual. **cobas c** 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

0.08-3.12 mmol/L (3-121 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:2 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 2.

Lower limits of measurement

Lower detection limit

0.08 mmol/L (3 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values

	No risk	Moderate risk	High risk
Females[28,29,30]	> 1.68 mmol/L (> 65 mg/dL)	1.15-1.68 mmol/L (45-65 mg/dL)	< 1.15 mmol/L (< 45 mg/dL)
Males[28,29,30]	> 1.45 mmol/L (> 55 mg/dL)	0.90-1.45 mmol/L (35-55 mg/dL)	< 0.90 mmol/L (< 35 mg/dL)

National Cholesterol Education Program (NCEP) guidelines:[31]

< 40 mg/dL: Low HDL-cholesterol (major risk factor for CHD)

≥ 60 mg/dL: High HDL-cholesterol ("negative" risk factor for CHD)

HDL-cholesterol is affected by a number of factors, e.g. smoking, exercise, hormones, sex and age.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

National Cholesterol Education Program (NCEP) guidelines are based on serum values, and when classifying patients, serum or serum equivalent values should be used. Therefore the NCEP recommends a factor of 1.03 to convert EDTA plasma values to serum values. However, our own investigations revealed that a factor of 1.06 should be used for the HDLC3 reagent. To comply with the 1998 NCEP goal of a < 5 % bias we recommend that each laboratory validate this conversion factor and enter it into the test parameters for HDL-C.[32]

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

Repeatability	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Precinorm L	1.38 (53.4)	0.01 (0.4)	0.4
Precipath HDL/LDL-C	0.89 (34.4)	0.01 (0.4)	1.0
Human serum 1	1.20 (46.4)	0.01 (0.4)	0.6
Human serum 2	2.08 (80.4)	0.01 (0.4)	0.7
Intermediate precision	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Precinorm L	1.34 (51.8)	0.01 (0.4)	0.9
Precipath HDL/LDL-C	0.88 (34.0)	0.01 (0.4)	1.5
Human serum 3	1.17 (45.2)	0.01 (0.4)	0.9
Human serum 4	2.03 (78.5)	0.02 (0.8)	0.9

Method comparison

HDL-cholesterol values for human serum and plasma samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (y) were compared with those determined using the corresponding reagent on a Roche/Hitachi MODULAR P analyzer (x).

Sample size (n) = 75

Passing/Bablok[33]	Linear regression
y = 1.000x + 0.000 mmol/L	y = 1.001x – 0.003 mmol/L
τ = 0.984	r = 0.999

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The sample concentrations were between 0.32 and 2.95 mmol/L (12.4 and 114 mg/dL).

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A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
→	Volume after reconstitution or mixing

FOR US CUSTOMERS ONLY: LIMITED WARRANTY

Roche Diagnostics warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label. THIS LIMITED WARRANTY IS IN LIEU OF ANY OTHER WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR PARTICULAR PURPOSE. IN NO EVENT SHALL ROCHE DIAGNOSTICS BE LIABLE FOR INCIDENTAL, INDIRECT, SPECIAL OR CONSEQUENTIAL DAMAGES.

CE

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com

Distribution in USA by:
Roche Diagnostics, Indianapolis, IN
US Customer Technical Support 1-800-428-2336

Roche

Appendix E: TG

002i7671073 22c501V9.0

TRIGL

cobas®

Triglycerides

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
20767107 322	Triglycerides 250 tests	System-ID 07 6710 7	**cobas c** 311, **cobas c** 501/502
10759350 190	Calibrator f.a.s. (12 x 3 mL)	Code 401	
10759350 360	Calibrator f.a.s. (12 x 3 mL, for USA)	Code 401	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
10171735 122	Precinorm U (4 x 5 mL)	Code 300	
12149435 122	Precinorm U plus (10 x 3 mL)	Code 300	
12149435 160	Precinorm U plus (10 x 3 mL, for USA)	Code 300	
10781827 122	Precinorm L (4 x 3 mL)	Code 304	
10171778 122	Precipath U (20 x 5 mL)	Code 301	
10171760 122	Precipath U (4 x 5 mL)	Code 301	
12149443 122	Precipath U plus (10 x 3 mL)	Code 301	
12149443 160	Precipath U plus (10 x 3 mL, for USA)	Code 301	
11285874 122	Precipath L (4 x 3 mL)	Code 305	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

TRIGL: ACN 781

For **cobas c** 502 analyzer:

TRIGL: ACN 8781

Intended use

In vitro test for the quantitative determination of triglycerides in human serum and plasma on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5,6]

Triglycerides are esters of the trihydric alcohol glycerol with 3 long-chain fatty acids. They are partly synthesized in the liver and partly ingested in food.

The determination of triglycerides is utilized in the diagnosis and treatment of patients having diabetes mellitus, nephrosis, liver obstruction, lipid metabolism disorders and numerous other endocrine diseases.

The enzymatic triglycerides assay as described by Eggstein and Kreutz still required saponification with potassium hydroxide. Numerous attempts were subsequently made to replace alkaline saponification by enzymatic hydrolysis with lipase. Bucolo and David tested a lipase/protease mixture; Wahlefeld used an esterase from the liver in combination with a particularly effective lipase from Rhizopus arrhizus for hydrolysis.

This method is based on the work by Wahlefeld using a lipoprotein lipase from microorganisms for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff (Trinder endpoint reaction). The color intensity of the red dyestuff formed is directly proportional to the triglyceride concentration and can be measured photometrically.

Test principle[6]

Enzymatic colorimetric test.

$$\text{triglycerides} + 3\ H_2O \xrightarrow{\text{LPL}} \text{glycerol} + 3\ \text{RCOOH}$$

$$\text{glycerol} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 2\ H_2O + HCl$$

Reagents - working solutions

R1 PIPES buffer: 50 mmol/L, pH 6.8; Mg^{2+}: 40 mmol/L; sodium cholate: 0.20 mmol/L; ATP: ≥ 1.4 mmol/L; 4-aminophenazone: ≥ 0.13 mmol/L; 4-chlorophenol: 4.7 mmol/L; lipoprotein lipase (Pseudomonas spec.): ≥ 83 µkat/L; glycerokinase (Bacillus stearothermophilus): ≥ 3 µkat/L; glycerol phosphate oxidase (E. coli): ≥ 41 µkat/L; peroxidase (horseradish): ≥ 1.6 µkat/L; preservative, stabilizers

R1 is in position B.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

Reagent handling

Ready for use

Storage and stability

TRIGL

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On board in use and refrigerated on the analyzer:	8 weeks

TRIGL

Triglycerides

cobas®

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.
Serum
Plasma: Li-heparin and K_2-EDTA plasma.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Stability:[7] 5-7 days at 2-8 °C
3 months at (-15)-(-25) °C
several years at (-60)-(-80) °C

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

See "Order information" section

General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma

cobas c 311 **test definition**

Assay type	1-Point	
Reaction time / Assay points	10 / 57	
Wavelength (sub/main)	700 / 505 nm	
Reaction direction	Increase	
Units	mmol/L (mg/dL, g/L)	
Reagent pipetting		Diluent (H_2O)
R1	120 µL	28 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	4 µL	15 µL	135 µL
Increased	2 µL	–	–

cobas c 501 **test definition**

Assay type	1-Point	
Reaction time / Assay points	10 / 70	
Wavelength (sub/main)	700 / 505 nm	
Reaction direction	Increase	
Units	mmol/L (mg/dL, g/L)	
Reagent pipetting		Diluent (H_2O)
R1	120 µL	28 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	4 µL	15 µL	135 µL
Increased	2 µL	–	–

cobas c 502 **test definition**

Assay type	1-Point	
Reaction time / Assay points	10 / 70	
Wavelength (sub/main)	700 / 505 nm	
Reaction direction	Increase	
Units	mmol/L (mg/dL, g/L)	
Reagent pipetting		Diluent (H_2O)
R1	120 µL	28 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	4 µL	15 µL	135 µL
Increased	4 µL	–	–

Calibration

Calibrators	S1: H_2O S2: C.f.a.s.
Calibration mode	Linear
Calibration frequency	2-point calibration - after reagent lot change - and as required following quality control procedures

Traceability: This method has been standardized against the ID/MS method.

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:

mmol/L x 88.5 = mg/dL	mmol/L x 0.885 = g/L
mg/dL x 0.0113 = mmol/L	mg/dL x 0.01 = g/L

TRIGL

Triglycerides

cobas®

Limitations - interference

Criterion: Recovery within ± 10 % of initial values at triglyceride levels of 2.3 mmol/L (203 mg/dL).

Icterus:[8] No significant interference up to an I index of 10 for conjugated bilirubin and 35 for unconjugated bilirubin (approximate conjugated bilirubin concentration: 171 µmol/L or 10 mg/dL; approximate unconjugated bilirubin concentration: 599 µmol/L or 35 mg/dL).

Hemolysis:[8] No significant interference up to an H index of 700 (approximate hemoglobin concentration: 434 µmol/L or 700 mg/dL).

Lipemia:[8] The L index correlates with sample turbidity but not with triglycerides level. Extremely lipemic samples (triglycerides greater than 3000 mg/dL) can produce normal results[9].

Prozone Check: The flag > Kin is an indicator for extremely high triglyceride concentrations in the sample. False normal results are due to oxygen depletion during assay reaction.

Endogenous unesterified glycerol in the sample will falsely elevate serum triglycerides.

Dicynone (Etamsylate) at therapeutic concentrations may lead to false-low results.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[10,11]

Exception: Ascorbic acid and calcium dobesilate cause artificially low triglyceride results. Intralipid is directly measured as analyte in this assay and leads to high triglyceride results.

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.[12]

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the carry-over evasion list can be found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1+2/SCCS Method Sheets. For further instructions refer to the operator's manual. **cobas c** 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

0.1-10.0 mmol/L (8.85-885 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:5 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 5.

Lower limits of measurement

Lower detection limit of the test

0.1 mmol/L (8.85 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values according to NCEP[13]

Normal range: < 1.70 mmol/L (< 150 mg/dL).

Clinical interpretation according to the recommendations of the European Atherosclerosis Society: [14]

	mmol/L	mg/dL	Lipid metabolism disorder
Cholesterol Triglycerides	< 5.18 < 2.26	< 200 < 200	No
Cholesterol	5.18-7.77	200-300	Yes if HDL-cholesterol < 0.9 mmol/L (< 35 mg/dL)
Cholesterol Triglycerides	> 7.77 > 2.26	> 300 > 200	Yes

Note: If the free glycerol is to be taken into account, the 0.11 mmol/L (10 mg/dL) must be subtracted from the triglycerides value obtained.[7]

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

	Repeatability		
	Mean mmol/L (mg/dL)	SD mmol/L (mg/dL)	CV %
Precinorm U	1.41 (125)	0.01 (1)	0.9
Precipath U	2.40 (212)	0.02 (2)	0.8
Human serum 1	1.67 (148)	0.02 (2)	1.1
Human serum 2	2.72 (241)	0.02 (2)	0.7

	Intermediate precision		
	Mean mmol/L (mg/dL)	SD mmol/L (mg/dL)	CV %
Precinorm U	1.39 (123)	0.03 (3)	2.0
Precipath U	2.33 (206)	0.04 (4)	1.6
Human serum 3	1.18 (104)	0.02 (2)	1.9
Human serum 4	2.95 (261)	0.05 (4)	1.8

Method comparison

Triglycerides values for human serum and plasma samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (y) were compared with those determined using the corresponding reagent on a Roche/Hitachi 917 analyzer (x).

Sample size (n) = 71

Passing/Bablok[15]	Linear regression
y = 1.015x - 0.005 mmol/L	y = 1.001x + 0.018 mmol/L
τ = 0.976	r = 0.999

The sample concentrations were between 0.560 and 9.13 mmol/L (49.6 and 808 mg/dL).

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Appendix F: DNA Extraction

The GUSTAFSON et al 1987 as modified by C Dandara et al was employed in for extraction and purification of DNA from 5ml frozen blood sample.

1) Frozen samples were thawed at room temperature and at times in a water bath at room temperature.

2) The sample was dispensed into 15ml appropriately labelled sterile screw top polypropylene tubes.

3) The sample tubes were rinsed with 1 ml PBS (Phosphate Buffered Saline) and the solution added to the polypropylene tube.

4) A 1:2 dilution was acievbed by adding with 2 volumes of PBS to the sample. There are many different ways to prepare PBS. Some formulations do not contain potassium, while others contain calcium or magnesium. Ours was based on the composition in the table below and ttis is the most common composition of PBS (1X). The simplest way to prepare a PBS solution is to use PBS buffer tablets or pouches. They are formulated to give a ready-to-use PBS solution upon dissolution in a specified quantity of distilled water. They are available in the standard volumes: 100, 200, 500 and 1000 mL. We used the 1000mL pouches.

Salt	Molecular Weight	Concentration (mmol/L)	Concentration (g/L)
NaCl	58.443	154	9
Na_2HPO_4	141.959	5.6	0.79
KH_2PO_4	136.086	1	0.13

pH was adjusted to 7.4 with HCl

5) Mix by inverting the tubes. The phosphate buffered saline (PBS), a balanced salt solution provides the essential balanced salt solution is to maintain pH and osmotic balance as well as provide cells with water and essential inorganic ions. It maintains cells in a viable state for short periods during the processing periods outside their natural habitat. One of the early formulas of PBS was developed by Renato Dulbecco, published in 19541 which are termed DPBS for Dulbecco's phosphate buffered saline.

6) The samples were centrifuged at 2200g (2300 rpm) for 15 minutes

7) The supernatant was carefully poured out leaving a reddish pellet at the bottom.

8) The pellet was resuspended in 10mL of Sucrose Triton X-100 Lysing Buffer and vortex.

The addition of sucrose produces a hypertonic solution which results in cell crenation tghus producing an increased surface area for the T20E5.

$MgCl_2$ in Triton offers DNA protection during subsequent steps. When membranes are busted by TRIS, there is no compartmentalization in the solution anymore. $MgCl_2$ from te Triton is already present to bind to DNA and thus protecting it against DNase proteins that will alsoi be released when the membranes are broken. The binding of $MgCl_2$ to DNA denies access of DNase to the DNA from being broken down.

Triton X-100 is a nonionic hydrophilic surfactant/detergent that is commonly used in laboratories to lyse cells to extract protein or organelles, or to permeabilize the membranes of living cells.

9) Place on ice for 5 minutes. This part is optional but it gives one a chance to review (take stock) the next steps and and also take a break as the below zero temperature slows down all possible metabolic activities.

10) Samples were spun for for 15 minutes or longer at 2200g (2300 rpm in TH.4 rotor)

11) The supernatant was carefully poured off leaving pellets that ranged from pink to white in colour.

12 Mixing by inversion is required after each of the following three steps.

12. i) The pellet was resuspended in 3 mL of TRIS20EDTA5 (T20E5) (The 3ml is calculated from the initial blood volume of 5ml X 0.6 =3mL. This is the first step in cell lysis TRIS (tris(hydroxymethyl)aminomethane):

When the cells are broken apart, their DNA and contents spill into the buffer. Additionally, RNase A (destroys RNA), proteases (destroys proteins), and SDS (sodium dodecyl sulfate, solubilizes the membrane fragments). All these components, fragmented RNA and proteins can have a big impact on the pH of the solution. DNA is pH sensitive, it is therefore important to ave a buffer can maintain the pH at a steady point. TRIS offers the optimum stable pH, usually 8.0 for DNA extraction, and its preference over other buffers is that it interacts with the lipopolysaccharides present on the outer membrane which helps to permeabilize the membrane. This effect is enhanced with the addition of EDTA (ethylenediaminetetraacetic acid), which binds divalent cations such as calcium and magnesium. These ions help maintain the integrity of the cell membrane and therefore, eliminating them with EDTA further destabilizes the membrane resulting in cell lysis.

12. ii) 200µl of 10% SDS was added producing a final concentration of 1%.

Sodium dodecyl sulfate (SDS), a strong anionic denaturing detergent that can solubilise proteins and lipids that form the membrane. It removes the -ve ions from the protein and destroys its confirmation. Loss of confirmation results in the protein losing its structure. This results in the cell membranes and nuclear envelopes to break down and expose the chromosomes that contain the DNA.In addition to removing the membrane barriers, SDS helps release the DNA from histones and other DNA binding proteins by denaturing them.

12. iii) 37.5µl Protenase K, 20mg/mL to final concentration of 250µg/mL –

The lypholised proteinase K is reconstituted wit 12.5ml of deionised water to give a concentration of 20mg/mL

The desired enzyme concentration in the final volume of 3ml (from 12i) is 250ug/mL but te available concentration is 20mg/mL

ViCi=V2C2

X x 20mg/mL =3ml x 250ug/mL

X x 20000ug/mL = 3ml x 250ug/mL

X = 3ml x 250/20000

X = 0.0375ml □ 0.0375 x 1000 = 37.5ul

Therefore 37.5ul of the reconstituted enzyme when added to 3ml gives 250ug/ml

Proteinase K (EC 3.4.21.64, protease K, Tritirachium album serine proteinase is a broad-spectrum serine protease. The enzyme was discovered in 1974 in extracts of the fungus Engyodontium album (formerly Tritirachium album). Proteinase K is able to digest native keratin (hair), hence, the name "Proteinase K". The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. It is commonly used for its broad specificity. This enzyme belongs to Peptidase family S8. The molecular weight of Proteinase K is 28,900 daltons (28.9 kDa).

Enzyme activity

Activated by calcium (1–5µmoles), the enzyme digests proteins preferentially after hydrophobic amino acids (aliphatic, aromatic and other hydrophobic amino acids). Although calcium ions do not affect the enzyme activity, they do contribute to its stability. Proteins will be completely digested if the incubation time is long and the protease concentration high enough. Upon removal of the calcium ions, the stability of the enzyme is reduced, but the proteolytic activity remains. Proteinase K has two binding sites for Ca^{2+}, which are located close to the active center, but are not directly involved in the catalytic mechanism. The residual activity is sufficient to digest proteins, which usually contaminate nucleic acid preparations. Therefore, the digestion with Proteinase K for the purification of nucleic acids is usually performed in the presence of EDTA (inhibition of calcium-dependent enzymes such as nucleases).

Proteinase K is also stable over a wide pH range (4–12), with a pH optimum of pH 8.0. An elevation of the reaction temperature from 37 °C to 50–60 °C may increase the activity several times, like the addition of 0.5–1% sodium dodecyl sulfate (SDS).

13) The preparation was incubated at 45°C overnight.

14) After incubation 1mL of saturated NaCl was added and mixed vigorously for 15 seconds

NaCl provides Na+ ions that will block negative charge from phosphates on DNA. Negatively charged phosphates on DNA cause molecules to repel each other. The Na+ ions will form an ionic bond with the negatively charged phosphates on the DNA, neutralizing the negative charges and allowing the DNA molecules to come together. Negatively charged phosphates on DNA cause molecules to repel each other neutralizing the negative charges and allows the DNA molecules to come together

15) The prepartation was spin for 30 minutes at 2400g. A white pellet composed of protein precipitated by salt should was visible.

16) The supernatant containing DNA was transferred to appropriately labellede new 15ml sterile screw top polypropylene tubes.

17) In order to precipitate the DNA, two volumes (approximately 9ml) of absolute alcohol kept at room temperature were added.

18) Several inversions approximately 50 times results in DNA precipitation. Interactions between two charges within a solvent are governed by Coulomb's law. Ethanol is the most commonly used alcohol component in precipitations of DNA and RNA. DNA is polar due to its highly charged phosphate backbone. Its polarity makes it water-soluble. Electric force which normally holds salt crystals together by way of ionic bonds is weakened in the presence of water allowing ions to separate from the crystal and spread through solution.

Ethanol has a much lower dielectric constant than water does (24 vs 80, respectively). By lowering the dielectric constant of a solution containing nucleic acids and monovalent cations, the Coulomb force of attraction increases between the cations and the negatively charged nucleic acid backbone

If enough ethanol is added, the electrical attraction between phosphate groups and any positive ions present in solution becomes strong enough to form stable ionic bonds and DNA precipitation causing it to separate out of solution. This usually happens when ethanol composes over 64% of the solution.

19) The preparation was spun for 10 minutes at at 2400g.

20) 1 mL of 70% ice-cold alcohol was used to wash the DNA. The 30% water allows partial dissolution of DNA and permits adhesion to the plastic wall of the tube.

21) The tubes were spun for 10 minutes at 2400g and the supernatant was removed carefully ensuring that there was no DNA adhering on the sides of the tubes.

22) Tubes were places mout down at 45° angle to air dry the DNA overnight.

23) DNA was redissolved in the approximate volume of TE (50-100µL)

TE buffer is a commonly used buffer solution in molecular biology, especially in procedures involving DNA, cDNA or RNA. "TE" is derived from its components: Tris, a common pH buffer, and EDTA, a molecule that chelates cations like Mg2+. The purpose of TE buffer is to solubilize DNA or RNA, while protecting it from degradation. TE buffer is also called as T10E1 Buffer, and read as "T ten E one buffer". To make a 100-ml solution of T10E1 Buffer, 1 ml of 1 M Tris-HCl (pH 8.0) and 0.2 ml EDTA (0.5 M) and made up with double distilled water up to 100ml. Based on nuclease studies from the 1980s, the pH is usually adjusted to 7.5 for RNA and 8.0 for DNA

The respective DNA and RNA nucleases are supposed to be less active at these pH values, but pH 8.0 can safely be used for storage of both DNA and RNA. EDTA further inactivates DNAse, by binding to metal cations which act as cofactors and therefore required by this enzyme. Genomic and plasmid DNA can be stored in TE Buffer at 4°C (39.2°F) for short-term use, or -20°C (-4°F) to -80°C (-112°F) for long-term storage.

24. The DNA in TE was transferred to sterile DNA cryotubes. See Figure below

Isolated DNA Storage preparation

DNA in TE was transferred to sterile DNA cryotubes

DNA concentration measurement

DNA concentration and quality was analysed on Nanodrop spectrophotometer (Thermo Scientific ND-1000). For each sample, 1.5 µl of dissolved DNA was loaded on the spectrophotometer, which provided the DNA concentration in ng/µl (values above 100 ng/µl were considered optimal), the 260/230 ratio determines the organic chemicals and solvent contamination. It is important to have this ratio above 1.6 for qPCR. This was very useful wen chloroporm was used as to precipitate te DNA. The 260/280 ratio determines protein contamination. Ratios above 1.8 reflect minimal proten contamination due to incomplete digestion. This is usually encountered due to subopimal temperature, inadequate incubation and unsufficient enzyme concentration. Samples were stored as stock DNA and working DNA was prepared for genotyping and stock DNA stored at −20°C prior to genotyping.

DNA 1% Integrity Assessment

Gel Preparation

1) 1g/mL of Seakem LE Agarose (1%W/V) was weighed using a triple beam balance.

2) The powder was placed in a graduated conical flask

3) Appropriate volume of 1x TBE (Tris Borate EDTA) buffer was added (e.g. 100mL 1xTBE and 1g Agarose)

Preparation of Stock 2L of 10xTBE

- 218 g Tris base
- 110 g Boric acid
- 9.3 g EDTA

Dissolve the ingredients in 1.9 L of distilled water. pH to about 8.3 using NaOH and make up to 2 L

(TAE –Tris Acetate EDTA is alsdo used by otherc laboratories.)

4) The solution was placed in a microwave. The agarose solution can boil over very easily so it was checked regularly. Usually we stopped it after 45 seconds to swirl. Oven gloves were worn during the removal swirling of the conical flask.

5) Once fully dissolved, the solution was clear and transparent and allowed to cool down by placing it in a container with water at room temperature and gently swirling the solution.

6) When the solution was cool enough to touch, Gel Green stain was added based on the 5ul Gel Green to 100mL of the gel solution proportion. Gel Green a proprietary dye is less toxic than ethidium bromide. Proprietary dyes bind to DNA and are fluorescent, meaning that they absorb invisible UV light and transmit the energy as visible light.

7) While the agarose was cooling, the gel tanks were prepared. The Gel solution was poured into a small 8x10 cm gels (minigels) which give good photographs because we found that larger gels did not good pictures.

8) The gel was poured gel slowly into the tank with a comb in position avoiding any bubbles.

9) The conical flask was rinsed immediately.

10) The gel was left to set for at least 30 minutes, preferably 1 hour, with the lid on if possible.

11) After setting, 1 x TBE buffer, the running buffer was added to the gel tank to submerge the gel to 2–5 mm depth.

Sample preparation

12) A para film was laid onto a smooth even bench surface and labelled from left to right with appropriate DNA sample numbers.

13) In front of each number 3µl of 1x loading buffer/dye was dispensed. Loading dye/buffers

give colour and density to the sample to make it easy to load into the wells and settle at the bottom. The dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. The colour allows visualising the progress of the gel

The most common dyes are bromophenol blue (Sigma B8026) and xylene cyanol (Sigma X4126). Density is provided by glycerol or sucrose. The loading dye was prepared as: i- 25 mg bromophenol blue or xylene cyanol

ii- 4 g sucrose

iii- H_2O to 10 mL

14) Equal volume of DNA samples 3µl were dispensed onto the corresponding loading buffer spot and gently mixed with the pipette tip and the mixture was dispensed into the corresponding gel well from left to right.

15) 3µl of the molecular weight marker (size marker) was loaded into the first and last wells)

Fermentas GeneRuler 100bp DNA Ladder containing 14 discrete fragments in base pairs ranging from 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 was utilised as molecular marker

Gel Running

16) An agarose gel was placed in this buffer-filled gel tank and an electrical field is applied via the power supply to the rear. DNA migrates from negative –cathode (black wire at the far end) towards positive –anode (red wire).

17) To avoid heating up the gel we utilised the principle of 5V/cm when running the gel (if the electrodes are 10 cm apart then run the gel at 50 V), we set ours at 100V and ran for 40 minutes.

Above 5 V/cm the agarose may heat up and begin to melt. Slower rate 2 V/cm for 10 minutes to allow the DNA to move into the gel slowly and evenly, and then speed up the gel later gives better resolution

18) An agarose gel is placed in this buffer-filled box and an electrical field is applied via the power supply to the rear. DNA migrates from negative –cathode (black wire at the far end) towards positive –anode (red wire). To check that the current was running we looked for evolving gas bubbles at the electrodes.

Viewing the gel

19) After switching off and unplugging the gel tank, the gel was carried in it's holder to the UV Viewing apparatus.

20) Tech Fire the gel (in its holder if possible) to the dark-room to look at on the UV light-box.

21) The viewing apparatus UV Tech Fire Reader was utilised.

2) Gel holders are not UV transparent the gel was placed onto the glass surface (dock) and preview was selected to edit the image with camera dials under white light.

23) UV Dock door was closed, white light switched off, then preview was also stopped and te UV light was switched on.

Note UV is carcinogenic and must not be allowed to shine on naked skin or eyes.

24) Exposure was selected and adjusted until optimum image was obtained.

Appendix G: Apo E RFLP Digest

AGGGGGAGCCCTATAATTGGACAAGTCTGGGATCCTTGAGTCCTACTCAGCCCCAGCGGA

GGTGAAGGACGTCCTTCCCCAGGAGCCGGTGAGAAGCGCAGTCGGGGGCACGGGGATGAG

CTCAGGGGCCTCTAGAAAGAGCTGGGACCCTGGGAACCCCTGGCCTCCAGGTAGTCTCAG

GAGAGCTACTCGGGGTCGGGCTTGGGGAGAGGAGGAGCGGGGGTGAGGCAAGCAGCAGGG

GACTGGACCTGGGAAGGGCTGGGCAGCAGAGACGACCCGACCCGCTAGAAGGTGGGGTGG

GGAGAGCAGCTGGACTGGGATGTAAGCCATAGCAGGACTCCACGAGTTGTCACTATCATT

TATCGAGCACCTACTGGGTGTCCCCAGTGTCCTCAGATCTCCATAACTGGGGAGCCAGGG

GCAGCGACACGGTAGCTAGCCGTCGATTGGAGAACTTTAAAATGAGGACTGAATTAGCTC

ATAAATGGAACACGGCGCTTAACTGTGAGGTTGGAGCTTAGAATGTGAAGGGAGAATGAG

GAATGCGAGACTGGGACTGAGATGGAACCGGCGGTGGGGAGGGGGTGGGGGGATGGAATT

TGAACCCCGGGAGAGGAAGATGGAATTTTCTATGGAGGCCGACCTGGGGATGGGGAGATA

AGAGAAGACCAGGAGGGAGTTAAATAGGGAATGGGTTGGGGGCGGCTTGGTAAATGTGCT

GGGATTAGGCTGTTGCAGATAATGCAACAAGGCTTGGAAGGCTAACCTGGGGTGAGGCCG

GGTTGGGGCCGGGCTGGGGGTGGGAGGAGTCCTCACTGGCGGTTGATTGACAGTTTCTCC

TTCCCCAGACTGGCCAATCACAGGCAGGAAGATGAAGGTTCTGTGGGCTGCGTTGCTGGT

CACATTCCTGGCAGGTATGGGGGCGGGGCTTGCTCGGTTCCCCCCGCTCCTCCCCCTCTC

ATCCTCACCTCAACCTCCTGGCCCCATTCAGGCAGACCCTGGGCCCCCTCTTCTGAGGCT

TCTGTGCTGCTTCCTGGCTCTGAACAGCGATTTGACGCTCTCTGGGCCTCGGTTTCCCCC

ATCCTTGAGATAGGAGTTAGAAGTTGTTTTGTTGTTGTTTGTTGTTGTTTTGTT

TTTTTGAGATGAAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAGTGGCGGGATCTCGGCT

CACTGCAAGCTCCGCCTCCCAGGTCCACGCCATTCTCCTGCCTCAGCCTCCCAAGTAGCT

GGGACTACAGGCACATGCCACCACACCCGACTAACTTTTTTGTATTTTCAGTAGAGACGG

GGTTTCACCATGTTGGCCAGGCTGGTCTGGAACTCCTGACCTCAGGTGATCTGCCCGTTT

CGATCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGCACCTGGCTGGGAGTTAGAG

GTTTCTAATGCATTGCAGGCAGATAGTGAATACCAGACACGGGGCAGCTGTGATCTTTAT

TCTCCATCACCCCCACACAGCCCTGCCTGGGGCACACAAGGACACTCAATACATGCTTTT

CCGCTGGGCGCGGTGGCTCACCCCTGTAATCCCAGCACTTTGGGAGGCCAAGGTGGGAGG

ATCACTTGAGCCCAGGAGTTCAACACCAGCCTGGGCAACATAGTGAGACCCTGTCTCTAC

TAAAAATACAAAAATTAGCCAGGCATGGTGCCACACACCTGTGCTCTCAGCTACTCAGGA

GGCTGAGGCAGGAGGATCGCTTGAGCCCAGAAGGTCAAGGTTGCAGTGAACCATGTTCAG

GCCGCTGCACTCCAGCCTGGGTGACAGAGCAAGACCCTGTTTATAAATACATAATGCTTT

CCAAGTGATTAAACCGACTCCCCCCTCACCCTGCCCACCATGGCTCCAAAGAAGCATTTG

TGGAGCACCTTCTGTGTGCCCCTAGGTACTAGATGCCTGGACGGGGTCAGAAGGACCCTG

ACCCACCTTGAACTTGTTCCACACAGGATGCCAGGCCAAGGTGGAGCAAGCGGTGGAGAC

AGAGCCGGAGCCCGAGCTGCGCCAGCAGACCGAGTGGCAGAGCGGCCAGCGCTGGGAACT

GGCACTGGGTCGCTTTTGGGATTACCTGCGCTGGGTGCAGACACTGTCTGAGCAGGTGCA

GGAGGAGCTGCTCAGCTCCCAGGTCACCCAGGAACTGAGGTGAGTGTCCCCATCCTGGCC

CTTGACCCTCCTGGTGGGCGGCTATACCTCCCCAGGTCCAGGTTTCATTCTGCCCCTGTC

GCTAAGTCTTGGGGGGCCTGGGTCTCTGCTGGTTCTAGCTTCCTCTTCCCATTTCTGACT

CCTGGCTTTAGCTCTCTGGAATTCTCTCTCTCAGCTTTGTCTCTCTCTCTTCCCTTCTGA

CTCAGTCTCTCACACTCGTCCTGGCTCTGTCTCTGTCCTTCCCTAGCTCTTTTATATAGA

GACAGAGAGATGGGGTCTCACTGTGTTGCCCAGGCTGGTCTTGAACTTCTGGGCTCAAGC

GATCCTCCCGCCTCGGCCTCCCAAAGTGCTGGGATTAGAGGCATGAGCCACCTTGCCCGG

CCTCCTAGCTCCTTCTTCGTCTCTGCCTCTGCCCTCTGCATCTGCTCTCTGCATCTGTCT

CTGTCTCCTTCTCTCGGCCTCTGCCCCGTTCCTTCTCTCCCTCTTGGGTCTCTCTGGCTC

ATCCCCATCTCGCCCGCCCCATCCCAGCCCTTCTCCCCGCCTCCCACTGTGCGACACCCT

CCCGCCCTCTCGGCCGCAGGGCGCTGATGGACGAGACCATGAAGGAGTTGAAGGCCTACA

AATCGGAACTGGAGGAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTCCA

AGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGCCGCC

TGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGG

TGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACC

TGCAGAAGCGCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCA

GCGCCATCCGCGAGCGCCTGGGGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCCACTG

TGGGCTCCCTGGCCGGCCAGCCGCTACAGGAGCGGGCCCAGGCCTGGGGCGAGCGGCTGC

GCGCGCGGATGGAGGAGATGGGCAGCCGGACCCGCGACCGCCTGGACGAGGTGAAGGAGC

AGGTGGCGGAGGTGCGCGCCAAGCTGGAGGAGCAGGCCCAGCAGATACGCCTGCAGGCCG

AGGCCTTCCAGGCCCGCCTCAAGAGCTGGTTCGAGCCCCTGGTGGAAGACATGCAGCGCC

AGTGGGCCGGGCTGGTGGAGAAGGTGCAGGCTGCCGTGGGCACCAGCGCCGCCCCTGTGC

CCAGCGACAATCACTGAACGCCGAAGCCTGCAGCCATGCGACCCCACGCCACCCCGTGCC

TCCTGCCTCCGCGCAGCCTGCAGCGGGAGACCCTGTCCCCGCCCCAGCCGTCCTCCTGGG

GTGGACCCTAGTTTAATAAAGATTCACCAAGTTTCACGCA

Area amplified by…: 218bp

ApoEF	TCCAAGGAGCTGCAGGCGGCGCA
ApoER	GCCCCGGCCTGGTACACTGCCA

TCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGGGCGCGGACATGGAGGACGTGTGCGGCCGCC

TGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGG

TGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACC

+

Appendix H: PCSK9 PCR and Sequencing

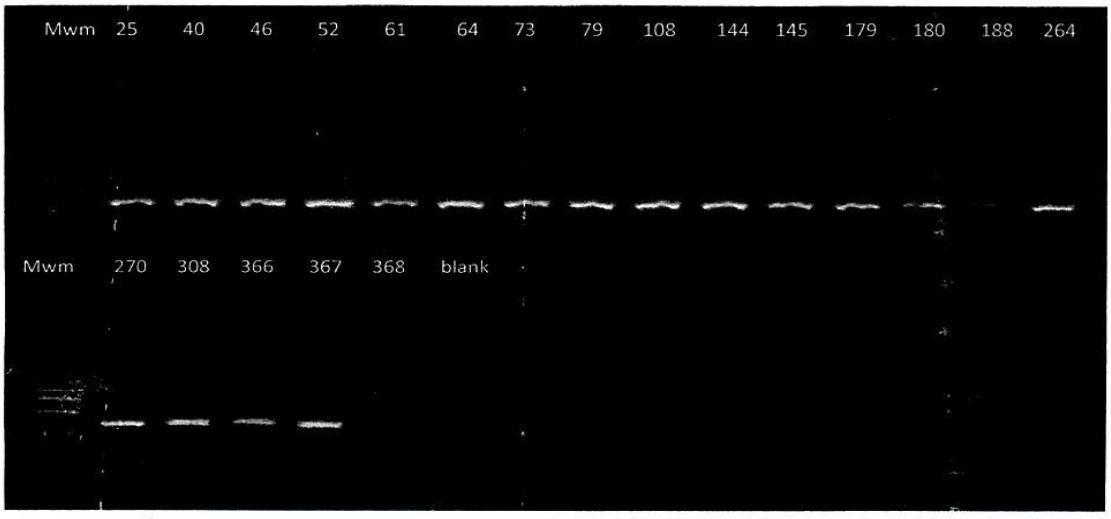
PCR amplification of PCSK9 rs505151

a) Add 1µL of 50ng/µL DNA to each PCR tube separately and change pipette tips at each stage
b) Prepare each PCR reaction MIX in a total volume of 25µL as follows:

Reagents	Volume used in a single reaction (in µL)	Volume for X reactions	Final conc. of reagents in PCR reaction
5X Green GoTaq reaction buffer	5		1X
5mM dNTPs	1		0.2mM
25mM $MgCl_2$	1.5		1.5mM
10µM Forward Primer	1		0.4µM
10µM Reverse Primer	1		0.4µM
5U/µL Fermentas Taq DNA polymerase	0.1		0.5U
Sterile distilled water (sdH_2O)	(Make up to 25 µL)		
Total reaction volume	25		

NB: Due to pipetting errors, for reactions up to 50, prepare enough for X+2, for reactions >50 prepare enough for X+5

a) Mix properly by tapping or pulse mix
b) Add 24µL of the PCR reaction mix to each PCR tube which already contains 1µL of DNA
c) PCR amplify using the "T100™ Thermal Cycler from Bio-Rad" with the following PCR cycling conditions:
    a) Denaturation at 95°C for 3min
    b) Denaturation at 95°C for 30s
    c) Annealing at 56.6°C for 30s
    d) Extension at 72°C for 30s
    e) Final extension at 72°C for 5min
    f) **Repeat steps b-d for 34 cycles
d) After completion of PCR, proceed with agarose gel electrophoresis by loading 5µL of PCR product on a 2% agarose gel stained with GRGreen (60min, 100V, 5µL Fermentas 100bp plus molecular weight marker)
e) Expected PCR product: 167bp DNA fragment
f) Check for quality of the PCR product by visualization on a UV-doc system. If successful proceed.



2% Agarose gel of PCSK9 rs505151 PCR amplification 4025-4368. 100V for 1hr

Clean-up of PCR products to remove primer and unincorporated dNTPs

a) Add 10µL of PCR product to each PCR tube separately and change pipette tips at each stage
b) Prepare the PCR clean-up reaction MIX in a total volume of 20µL as follows:

Reagents	Volume used in a single reaction (in µL)	Volume for X reactions	Final conc. of reagents in reaction
1U/µL FastAP	1	20	1U
20U/µL Exonuclease I	0.1	2	2U
Sterile distilled water (sdH_2O)	(Make up to 20µL)	178	
Total reaction volume	20		

NB: Due to pipetting errors, for reactions up to 50, prepare enough for X+2, for reactions >50 prepare enough for X+5

a) Mix properly by tapping or pulse mix
b) Add 10µL of the PCR clean-up reaction mix to each PCR tube which already contains 10µL of PCR product
c) PCR clean-up using the "T100™ Thermal Cycler from Bio-Rad" with the following cycling conditions:
    a) 37°C for 60min
    b) 75°C for 15min
d) After completion of PCR clean-up, proceed with cycle sequencing

Sequencing of cleaned-up PCR products to determine the nucleotide sequence of this fragment (including the SNP)

a) Add 3µL of cleaned-up PCR product to each PCR tube separately and change pipette tips at each stage
b) Prepare the sequencing reaction MIX in a total volume of 10µL as follows:

Reagents	Volume used in a single reaction (in µL)	Volume for X reactions	Final conc. of reagents in reaction
10µM Forward or Reverse Primer	1	17	1µM
BigDye Terminator Mix v.3.1	1	17	
5X Sequencing Buffer	2	34	1X
Sterile distilled water (sdH_2O)	(Make up to 10µL)		
Total reaction volume	10		

NB: Due to pipetting errors, for reactions up to 50, prepare enough for X+2, for reactions >50 prepare enough for X+5

a) Mix properly by tapping or pulse mix
b) Add 7µL of the PCR reaction mix to each PCR tube which already contains 3µL of cleaned-up PCR products
c) Sequencing amplify using the "GeneAmp PCR system 9700 from Applied Biosystems" with the following cycling conditions:
    a) Denaturation at 98°C for 5min
    b) Denaturation at 96°C for 30s
    c) Annealing at 50°C for 15s
    d) Extension at 60°C for 4min
    e) **Repeat steps b-d for 35 cycles
d) After completion of sequencing, proceed with ethanol precipitation:
    a) Transfer the sample to a new, labelled 1.5ml Eppendorf tube

b) Add 22µL cold 100% ETOH
c) Add 1µL 3M NaOAc pH5.2
d) Keep at -20°C overnight
e) Centrifuge at 10 000rpm for 10min, remove supernatant
f) Add 35µL ice-cold 70% ETOH, vortex and centrifuge at 10 000rpm for 10min
g) Remove supernatant and air-dry for at least 1hr
h) Resuspend pellet in 10µL HiDi

e) After completion of ethanol precipitation, proceed with capillary electrophoresis by using 5µL of sequencing product with 5µL of HiDi, vortex and denature on the PCR machine at 94°C for 5min
f) Perform electrophoresis on ABI 3130xl at Human Genetics, UCT
g) View, align and annotate sequencing results using DNAstar

Appendix I: Subject Information Document

Study title:

GENETICS AND DIABETIC DYSLIPIDAEMIA IN THE BLACK SOUTH AFRICANS

Greeting: Good day, my name is Donald Tanyanyiwa. I work in Chemical Pathology here at Chris Hani Baragwanath Academic Hospital

Introduction:

We are doing some work to help us understand causes of increased fat /lipids in some African people with diabetes. It is known that some people with DMdevelop abnormal levels of fats/lipids. People are born with fat carrying protein, which may be different because of changes (mutations/polymorphism) in the seed (genes) that produce them and that causes them to do their job differently; others do it faster than others. The change may occur in one seed or many seeds. This causes some people to have increased amounts of different types of fat because their fat carrying proteins will not be working properly or may be reduced. Fats/Lipids are required by the body, but when in excess it becomes harmful. The body normally manages to keep the fat within the required levels, but during illness or the presence of inherited disease, the body may not be able to clear the fat.

High amounts of these fats have been associated with disruption of the normal heart and blood transport system. A lot of research to identify the different causes of increased amount has been done but very little if any has been done to identify the common causes of fat accumulation in African people with diabetes. The study will examine to see if mutations/polymorphism in the genes of some (four proteins) associated with lipid metabolism are responsible for the abnormal fat/lipid (cholesterol and triglyceride) content in people with diabetes. The study will require only a small amount of blood (1.5 teaspoons) of the blood already sent to the laboratory for diagnostics purposes. There will be no immediate benefits to you but depending on the results benefits may be realised later. Patients who do not participate or withdraw from the study will continue to receive the same recommended standard intervention as the participants. It is hoped that the findings and advice given to the patients will go a long way in improving the quality of life. Venesection is a very safe method of obtaining blood for the repeat lipogram if required. The only risk that can be encountered is prolonged bleeding in those with some coagulopathy, a condition that will be excluded by the medical history on previous venesection.

Invitation to participate: We saw from your results that you are one of those diabetic people with increased amounts of these fats. Usually a small amount of blood (one and half (1½) teaspoons) is sent to test fat content in blood. We are asking your permission to keep that blood and find out the reason why the fat is increased

What is involved in the study – We will not need any blood from you and we will not examine you. However, we will require your permission to go and look at your hospital/clinic file to get your age, and how you are being treated. If you are taking tablets we will record their names. You will not be expected to make any special visit to Chris Hani Baragwanath Academic Hospital for this study.

Risks: There is no risk at all in this study because the fat carrying protein is already in the blood you sent to the laboratory and that is the one we will be using.

Benefits - The benefits of this study will only be realized if the cause of increased fat is identified and because it is the first such study, I will present it for as a PhD thesis.

Participation is voluntary – It is your right to accept or refuses to take part in this study and whatever decision you make nothing will change regarding your treatment. It is also your right to tell us to destroy the blood that is already in the laboratory.

Appendix J: Consent Form

CONSENT FOR STYDY ON DIABETIC DYSLIPIDAEMIA IN BLACK SOUTH AFRI-CANS

Name----------

Address----------

Analysis and storage of Biologic Samples: Plasma and DNA

I hereby consent to the removal, storage waiting processing, and analysis of the above material from my own body for the purpose of diagnosis and research into disorders of lipid and lipoprotein metabolism associated with diabetes. After due explanation I understand that

1) Conventional procedures and techniques are employed and that the health risk is minimal.

2) The material and results of the investigations remain strictly confidential according to medical practice and such ethical guidelines that govern research at the universities and country at large. To preserve anonymity, the samples are coded by numbers and my written consent is required for release of identifiable information to another party

3) Precise diagnosis may not always be possible because the defect(s) may not yet be known or there is inadequate information to derive the defect owing to modulatory roles that other genes or the environment may play.

4) The stored material may be used anonymously in future to derive information or for research purposes. Such future use may be of no direct benefit to the subject.

5) Permission to participate in the study may be withdrawn at any time and any stored biological material will also be destroyed. The withdrawal will not affect the subject's future medical care.

6) I confirm that the purpose of this study was explained in the language that I preferred and I fully understood it. I have not received any financial benefit and I do not expect to receive any in future and undertake not to make any future financial claims following the outcome and publica-tion of the finding.

Singed at ----------day ----------month---------2013

Print Name ---------- Signature----------

Witness (Subject's Choice)

Print Name ---------- Signature----------

Witness (Health Personnel)

Print Name ---------- Signature----------

Appendix K: Data Collection Sheet

DIABETIC DYSLIPIDAEMIA DATA COLLECTION SHEET

PROJECT NUMBER			**DDL------/2013**	
FOLDER NUMBER				
GENDER	**MALE**		**FEMALE**	
DATE OF BIRTH	**/ /**		**AGE**	**YEARS**
DATE IN DIABETIC CLINIC				

LIPOGRAM			
	DATE		
Total Cholesterol		**HDL-Cholesterol**	
Triglyceride		**LDL-Cholesterol**	

TYPE OF DYSLIPIDAEMIA			
1	**HYPERCHOLESTEROLAEMIA**		
2	**HYPERTRIGLYCERIDAEMIA**		
3	**MIXED**		

MUTATION OR POLYMORPHYSM IDENTIFIED	
ApoE	
PCSK9	