The association between vitamin D, vitamin D binding proteins and VDR polymorphisms in diabetic and non-diabetic patients

By

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Declaration

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Abstract

Introduction: Type 2 diabetes mellitus (T2DM) is by far the most prevalent form of diabetes manifesting with insulin resistance (IR), abnormal pancreatic β-cell function and hyperglycaemia. Evidence from epidemiological and observational studies have shown that vitamin D deficiency is associated with increased risk for T2DM although the findings are inconsistent and inconclusive. In the circulation vitamin D is transported bound to vitamin D binding protein (VDBP), evidence showed that vitamin D levels are positively associated with VDBP levels. Several genes such as vitamin D receptor gene (VDR), involved in the metabolic pathway of T2DM have been considered good candidate for susceptibility to T2DM. The present study aimed to investigate the association between vitamin D, vitamin D binding proteins (VDBP) and vitamin D receptor (VDR) polymorphisms in T2DM and non-diabetic patients in the mixed ancestry population.

Materials and methods: The current study comprised of 1603 participants (387 males and 1216 females). Vitamin D levels were measured using the paramagnetic particle chemiluminescence test on a Beckman DXI. Vitamin D binding protein (VDBP) in serum samples was measured using the Human Vitamin D BP Quantikine ELISA kit. Fok1 (*rs2228570*), Apa1 (*rs7975232*) and Taq1 (*rs731236*) single nucleotide polymorphisms (SNPs) of the VDR gene were genotyped from a genomic DNA using the TaqMan SNP Genotyping Assays and were confirmed by direct sequencing.

Results: Vitamin D deficiency (44%) and insufficiency (42.6%) were highly prevalent and optimal 25(OH)D levels were very low with only 13% having optimal levels. The overall vitamin D status of the whole population group was insufficient (22.0±7.6 ng/mL). 25(OH)D levels and serum VDBP varied according to gender with males having higher 25(OH)D levels (23.6±7 vs 21.5±7.5ng/mL, P=0.0006) and females with significantly higher serum VDBP levels (299.1±71.2 vs 315.9±76.1 µg/mL, P<0.0001). 25(OH)D levels were generally significantly decreased in the hyper-glycemic subgroups. Screen-detected DM males had low 25(OH)D levels compared to normo-glycaemic group (17.0±6.1vs 24.2±8.2, P=0.0214). A similar trend was observed in the female groups (21.1±6.0 vs 22.4±7.9, P=0007). Anthropometric measurements including the BMI (kg/m2), Waist C (cm) and Hip C (cm) were significantly higher in hyper-glycaemic group than in normo-glycaemic males and females (All, P<0.0001).

In contrast, there were no significant differences in serum VDBP (µg/mL) between the glycaemic sub-groups in either male (P=0.5614) or females (P= 0.4813). The glycaemic parameters, as expected, were significantly increased in the hyper-glycaemic sub-groups in both genders, including FBG (mmol/L), 2 hr BG (mmol/L), HbA1c (%), FBI (mIU/L), 2 hr BI (mIU/L) and HOMA-IR (All, both males and females P<0.0001). In general, the lipids, including the triglycerides (mmol/L), LDL-C (mmol/L) and Cholesterol (mmol/L) were also significantly increased in both genders in the hyper-glycaemic sub-groups (All, males P≤0.0300, females P<0.0001), while HDL-C (mmol/L) was significantly decreased in both males and females in the hyper-glycaemic sub-groups (All, P≤0.0308).

The variant genotype GG of the Fok1, AA of Apa1 and GG of the Taq1 SNPs were not significantly different in hyper-glycaemic patients compared to normo-glycaemic group (58.5% vs 55.1%, P-value, 40.1% vs 38.0%, P-value and 6.9% vs 8.5%, P-value,) respectively. Similarly, there was no significant difference in the alleles frequency distribution of these SNPs between the groups. Results also demonstrated no significance difference in the genotype or allele frequency distribution of Fok1 (rs2228570), Apa1 (rs7975232) and Taq1 (rs731236) SNPs between subjects with optimal Vitamin D (25(OH)D ng/mL) levels and those with insufficient/deficient levels (P≥0.2036 and P≥0.6347 respectively). These trends were also observed when serum VDBP levels were evaluated against Fok1, Apa1 and Taq1 genotypes.

Multiple linear regression showed that low 25(OH)D was associated with increased LDL-C and PTH in both male and females irrespective of T2DM, but serum VDBP was associated with low 25(OH)D in hyper-glycaemic females only. In normo-glycaemic males 19.5% of the variation in 25(OH)D was attributed to increased LDL-C and in the hyper-glycaemic group 15.5% it was attributed to PTH and CRP. In normo-glycaemic females 12.8% variation in 25(OH)D was attributed to LDL-C, serum creatinine and PTH, whereas in hyper-glycaemic group 16.1% was attributed to increased age, serum VDBP, triglycerides, LDL-C, creatinine and PTH.

Conclusion: This study showed prevalence of vitamin D deficiency/insufficiency in the mixed ancestry population group. There was no association between vitamin D (25(OH)D), vitamin D binding proteins (serum VDBP) and VDR polymorphisms in T2DM patients. Serum VDBP levels were associated with low vitamin D levels in

hyper-glycaemic females only. Increased LDL-C, PTH and CRP were predictors of low vitamin D levels.

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Abbreviations

ADA : American Diabetes Association

Albumin-S : Serum Albumin

ALT : Alanine Transferase

AST : Aspartate Transferase

BMI : Body Mass Index

Calcium-S : Serum Calcium

Chol : Cholesterol

Creatinine -S : Serum Creatinine

Creatinine-U : Urine Creatinine

CRP : C-Reactive Protein.

CVDs ; Cardiovascular Diseases

DCCT : Diabetes Control and Complications Trial

DHRC7 : 7-Dehydrocholesterol reductase

DM : Diabetes Mellitus

FBG : Fasting Blood Glucose

FBI : Fasting Blood Insulin

Gamma GT-S : Serum Gamma Glutamyl transferase

Gc : Group component

GDM : Gestational Diabetes Mellitus

HbA1c : Glycated Haemoglobin

HDL-C : High Density Lipoprotein Cholesterol

Hip C : Hip Circumference

HOMA-IR : Homeostasis Model Assessment for Insulin Resistance

IDF : International Diabetes Federation

IFG : Impaired Fasting Glucose

IGT : Impaired Glucose Tolerance

LDL-C : Low Density Lipoprotein Cholesterol

MDRD : Modification of Diet in Renal Disease

MODY : Maturity Onset Diabetes of the Young

NCDs : Non-Communicable Diseases

NGSP : National Glycohemoglobin Standardization Program

NHANES : National Health and Nutrition Examination Survey

OGGT : Oral Glucose Tolerance Test

Phosphate-S : Serum Phosphate

PTH : Parathyroid Hormone

SNPs : Single Nucleotide Polymorphisms

Sodium-S ; Serum Sodium

TC : Total Cholesterol

TG : Triglycerides

TMB : Tetramethylbenzidine

T1DM : Type 1 Diabetes Mellitus

T2DM : Type 2 Diabetes Mellitus

VDBP : Vitamin D binding Protein

VDR : Vitamin D Receptor

Waist C : Waist Circumference

WHR : Waist-Hip Ratio

WHO : World Health Organisation

2 hr BG	: Post 2 Hours Blood Glucose
2 hr BI	: Post 2 Hours Blood Insulin

Definition of concepts

Diabetes mellitus : Is a group of metabolic disorders characterized by raised glucose levels in the blood resulting from defects in insulin secretion, insulin resistance or both

Metabolic syndrome : Is a cluster of metabolic disorders occurring at ones thus increasing risk for Type 2 diabetes mellitus and cardiovascular diseases.

Insulin resistance : Is a pathological condition in which the body cells do not respond to the effects of hormone insulin

Vitamin D deficiency : Is defined as vitamin D levels below 20ng/ml in the blood circulation.

Vitamin D insufficiency : Is defined as vitamin D levels below above 20ng/ml but below 30ng/ml in the blood circulation

Vitamin D : Is a lipid soluble vitamin responsible for maintenance of body mineral homeostasis and bone health

Vitamin D Binding Protein : Is a glycoprotein responsible for transporting vitamin D in the circulation to vitamin D requiring cells.

Vitamin D receptor : Is a steroid/thyroid hormone receptor superfamily that functions as a transcriptional activator of many genes.

Chapter 1 Introduction

The frequency of diabetes mellitus is rapidly increasing globally. More recently, the International Diabetes Federation (IDF) estimates shows that 415 million (uncertainty: 340-536 million) people aged 20-79 years had diabetes in the year 2015 (Ogurtsova et al., 2017). Diabetes was accountable for about 5.0 million deaths. About a quarter (75%) of these diabetic cases resides in the low-and middle-income countries (LMICs). This number of type 2 diabetes cases is projected to rise to 642 million (uncertainty: interval of 521-829 million) in 2040. Type 2 diabetes mellitus (T2DM) is the most prevalent form of diabetes manifesting with insulin resistance, abnormal pancreatic β-cell function and hyperglycaemia (Takiishi et al., 2010). T2DM is a major cause of morbidity and mortality accounting for over 90% diabetes cases globally. Several genetic and environmental factors have been implicated in its onset and progression.

It is known that pathophysiology of T2DM involves impaired insulin secretion with a coexisting insulin resistance (Pittas et al., 2010). Studies have shown that high vitamin D levels can enhance pancreatic β-cell function and improve insulin resistance (Pittas *et al.*, 2010); (Ozfirat et al., 2010). Vitamin D exerts its cellular functions through binding Vitamin D receptor (VDR) (Al-Daghri et al., 2012), an intracellular hormone receptor which belongs to steroid hormone receptor superfamily (Wang et al., 2012). Thus, VDR gene is considered an important candidate gene for susceptibility to type 2 diabetes mellitus (T2DM) (Abdeltif et al., 2014). Furthermore, genetic alterations in the VDR gene may lead to defects in gene activation or alter protein function/structure of which could affect both the binding and affinity of vitamin D and its functions.

Currently, Fok1, Bsm1, Apa1 and Taq1 single nucleotide polymorphisms (SNPs) of the VDR gene are the commonly studied VDR polymorphisms in relation to T2DM susceptibility, cancers, autoimmune and infectious diseases (Uitterlinden et al., 2004). Correlations between VDR polymorphisms and parameters associated with T2DM such as glucose intolerance, insulin insensitivity, altered insulin secretion and vitamin D deficiency have been reported (Valdivielso et al., 2006). Numerous studies have examined the association between these four polymorphisms and T2DM risk. However, their results were inconsistent and inconclusive across various ethnic populations.

There is no available data on the association of vitamin D, vitamin D binding proteins and VDR polymorphisms in the mixed ancestry population of Bellville South, Cape Town, South Africa despite a high prevalence (28%) of T2DM being reported in this population group (Erasmus et al., 2012).Therefore, the current study aims to examine the association between vitamin D levels, vitamin D binding proteins and VDR polymorphisms in diabetic and non-diabetic patients within the mixed ancestry population-group.

Chapter 2 Literature review.

2.1 Definition of diabetes.

Diabetes mellitus (DM) is defined as a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion and insulin action or both (American Diabetes Association, 2014). The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction and failure of various organs mainly the eyes, kidneys, nerves, heart and blood vessels (American Diabetes Association, 2014). Several pathogenic processes are implicated in the onset and progression of diabetes. These include autoimmune destruction of the pancreatic β-cells resulting in insulin deficiency or inadequate insulin action to target cells or tissues (American Diabetes Association, 2014).

2.2 Classification of diabetes mellitus.

Diabetes mellitus is classified into two major types: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus(T2DM). Other less common types include gestational diabetes mellitus (GDM) and maturity-onset of the young (MODY) and latent diabetes in adults (LADA).

2.2.1 Type 1 diabetes mellitus (T1DM).

The pathophysiology of T1DM involves autoimmune destruction of pancreatic β-cells thus resulting in absolute insulin deficiency and hyperglycaemia (Mohammadnejad et al., 2012);(Gregory et al., 2013). T1DM is formerly known as insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes mellitus. Immune destruction of the pancreatic β-cells is marked by presence of the autoantibodies of islet cells, insulin autoantibodies and autoantibodies to the glutamic acid decarboxylase 65 (GAD65). These abovementioned autoantibodies are present after the initial detection of hyperglycaemia. (Amercan Diabetes Association, 2010).

This disease predominantly affects children, adolescents and adults aged below 30 years old (Mohammadnejad *et al.*, 2012). It accounts for about 5-10% of cases with diabetes mellitus and majority of diagnosed cases are children. Lack of insulin lead to dysregulation of glucose levels in the body, thus limiting glucose transport to the target cells. Hence, that leads to increased gluconeogenesis and lipolysis, which in turn results with the formation of ketone bodies (acetoacetic acid and β-hydroxybutyric acid

and acetone) from lipolysis (Gregory *et al.*, 2013). Accumulation of these metabolites in blood circulation lead to development of diabetic ketoacidosis (DKA). DKA is the major cause of death in children suffering from T1DM (Cooke et al., 2008).

2.2.2 Type 2 diabetes mellitus (T2DM).

T2DM Formerly known as non-insulin dependent diabetes mellitus (NIDDM) or adult onset diabetes mellitus is characterized by raised blood sugar levels or hyperglycaemia arising from two pathological conditions, namely: insulin resistance and β- cell dysfunction (Holt et al., 2004); (Cerf et al., 2013) . Studies have reported that obesity has led to a dramatic increase in the incidence of T2DM more especially among children and adolescents (Haemer et al., 2014). In addition, obesity is strongly associated with insulin resistance of which when coupled with insulin deficiency it leads to an overt T2DM (Haemer *et al.*, 2014). Insulin resistance refers to a condition characterized by impaired ability of the insulin to transport glucose to the target cells, whilst β- cell dysfunction refers to suboptimal or insufficient secretion of insulin from the β-cells (Holt et al., 2004). The above-mentioned disorders disturb the maintenance of glucose homeostasis. Accumulating evidence showed that insulin resistance and β-cell dysfunction predicted the development of T2DM independently of known and unknown risk factors.

2.2.3 Gestational diabetes mellitus (GDM).

Gestational diabetes mellitus is an overt diabetes that occurs in up to 2-5% of pregnant women during pregnancy. It can thus lead to serious complications for the mother and child and they are both at high risk for developing T2DM at later life (Ben-Haroush et al., 2004); (Chu et al., 2007). Complications include preeclampsia or hypertension during pregnancy, premature birth and respiratory distress syndrome and still birth or dead foetus (Bodnar et al., 2010). Risk factors for GDM include obesity, previous history of GDM, advanced age≥ 25 years old and family history of diabetes (Zhang et al., 2011).

In addition, a meta-analysis found that high maternal weight (overweight and obesity) is associated with substantially increased risk for GDM as compared to lean women (Chu *et al.*, 2007). Also increased insulin resistance and lack of physical activity contributes to increased risk for GDM. GDM is caused by failure of the insulin action to regulate glucose levels during pregnancy thus resulting in hyperglycaemia in the

circulation and insulin resistance (Barbour et al., 2007). Insulin resistance during pregnancy is also caused by the effect of substances released by placenta which tempers with the normal function of insulin (Barbour *et al.*, 2007).

2.2.4 Maturity-onset diabetes of the young (MODY).

Maturity-onset diabetes of the young is a very rare form of T2DM, characterized by hyperglycaemia, impaired insulin secretion with minimal or no defects in insulin action due to mutations in insulin genes (American Diabetes Association, 2014). This disease is inherited in an autosomal dominant pattern, thus it accounts for 1-2% of all diabetic cases (Shields et al., 2010). MODY patients are often misdiagnosed as T1DM/T2DM. This disease is also characterized by its early onset at early childhood, adolescent or age below 25 years.

2.2.5 Latent Autoimmune Diabetes in Adults (LADA).

Latent autoimmune diabetes of the adults (LADA) is an autoimmune diabetes defined by adult-onset ≥ 35 years, presence of diabetes associated autoantibodies (DAA), and no insulin treatment requirement for a period after diagnosis (Laugesen et al., 2015). LADA accounts for about 12% of all diabetic cases in adult populations (Naik et al., 2009). Common DAA includes glutamic acid decarboxylase 65 (GAD65), insulinoma antigens IA-2 (IA-I2), islet cells and zinc transporter 8 (Lampasona et al., 2010). Immunologically GAD65 is the most prevalent form of autoantibody presence in adult onset diabetes. This disease shares genetic features with both T1DM and T2DM. LADA patients are often misdiagnosed as T2DM, due to similar phenotypic appearance and disease age-onset (Appel et al., 2009). Moreover, LADA patients have worse HbA1c levels as compared to T2DM. accumulating evidence have shown LADA tend to have lower mean age at onset, lower BMI and more frequent need for insulin treatment than T2DM patients (Laugesen *et al.*, 2015). Patients with LADA have slow β- cell destruction thus insulin treatment is not required at the time of diagnosis (Appel *et al.*, 2009).

2.3 Risk factors for type 2 diabetes mellitus (T2DM).

Type 2 diabetes (T2DM) is a multifactorial disease which arises from the complex interaction of both modifiable and non-modifiable risk factors. These risk factors may range from genetic level to environmental.

2.3.1 Modifiable risk factors.

2.3.1.1 Obesity and body fat distribution

Obesity is one of the modifiable risk factors for T2DM, it has been extensively studied. It is defined as body mass index (BMI) ≥ 30 kg/m^2, this unit of measure has been traditionally used to determine prevalence of obesity in national population based studies (Nguyen et al., 2010). It has been reported that the increase in obesity has been accompanied by an increasing prevalence of T2DM. Since obesity is such a strong predictor of T2DM incidence, then the higher prevalence of T2DM reported among different populations previously is almost certainly attributed to an increase in obesity rates. A meta-analysis has shown a strong association between measures reflecting abdominal obesity such as waist circumference (WC) and the development of Type 2 diabetes (Freemantle et al., 2008). It is therefore assumed that reducing WC may reduce the development of T2DM.

Abdominal obesity is known as the combination of subcutaneous and visceral fat and has been widely reported as risk factors for T2DM and is also associated with a poor metabolic profile (Freemantle *et al.*, 2008). The association between increased abdominal obesity and T2DM can be partly attributed to the increased release of non-esterified fatty acids (NEFA) and production of pro-inflammatory cytokines from the abdominal fat depot (Karpe et al., 2011). Higher NEFA and production of pro-inflammatory cytokines are believed to alter insulin signalling thus resulting in insulin resistance (Kahn et al., 2006); (Karpe *et al.*, 2011). Furthermore, production of these metabolites increases with the degree of obesity.

2.3.1.2 Physical Activity (PSA)

Engagement in physical activity is the most recommended key factor for the prevention and management of T2DM, Hence both obesity and increased sedentary lifestyle are considered as risk factors for the development of T2DM (Gajardo et al., 2017) with the former being a higher contributor to T2DM risk compared to physical inactivity (Rana et al., 2007)

According to WHO, insufficient PSA is defined as less 150 minutes of moderate physical activity per week or equivalent. In Africa about a quarter of men and women presented with insufficient PSA. A study from Iran reported that moderate physical activity ≥150 min/week was associated with reduced risk for T2DM in all non-obese

people, however in obese such an effect was not observed (Ghaderpanahi et al., 2011). In a multi-ethnic study of atherosclerosis (MESA), the incidence type 2 diabetes was significantly and inversely associated with exercise and vigorous physical activity, typical walking pace, and conversely associated with sedentary lifestyle (Joseph et al., 2016). This indicates that moderate to vigorous intensity physical activity may reduce the risk for development of T2DM and in obese individuals moderate physical activity should be increased.

2.3.1.3 Poor diet.

Diet is considered as a modifiable risk factor for T2DM. Accumulating evidence have shown that high consumption of refined carbohydrates, saturated and trans fats is associated with an increased risk for T2DM by adversely affecting glucose metabolism and insulin resistance (Hu et al., 2001).Furthermore, evidence also suggests that consumption of different micronutrients could contribute to increased risk for development of T2DM. Studies have shown that a high intake of food rich in fibre such as whole grain-cereals contributes to reduction in HOMA-IR and lower prevalence of metabolic syndrome (McKeown et al., 2004). Moreover, a multi-ethnic cohort study reported that food high in meat and fat confers higher diabetes risk in all ethnic groups, although the effects of other dietary patterns substantially differ by sex and race/ethnicity (Erber et al., 2009).In addition, evidence demonstrates that beneficial effect of the micronutrient such as vitamin D is not limited to bone health but also to non-skeletal diseases including cancer, autoimmune disorders, cardiovascular disease and T2DM (Engelman et al., 2010). The protective role of vitamin D against the development of T2DM will be discussed in detail later.

2.3.1.4 Hypertension.

In the literature there is considerable evidence for an increased prevalence of hypertension in diabetic patients from other populations have shown a sharp increase in the prevalence of hypertension in diabetic patients (Berraho al., 2012). According to ADA guidelines (2018), hypertension is defined as a sustained systolic blood pressure of (≥140 mmHg) over diastolic blood pressure of (≥90 mmHg). Both diabetes and hypertension predispose patients to the development of CVDs and renal diseases. For example, prospective cohort study comprised of 12550 adults have shown that the development of T2DM was almost 2.5 times as likely in persons with hypertension than in their normotensive counterparts (Berraho al., 2012);(Sowers et al., 1995).

Furthermore, the age adjusted relative risk of death consequent to cardiovascular events in T2DM patients is 3-fold higher than in the general population (El-Atat et al., 2004). Additionally, the presence of hypertension in T2DM patients substantially increases the risk of coronary heart diseases, stroke, neuropathy and retinopathy. Therefore, blood pressure control is vital and necessary for prevention of the development of T2DM and CVDs in the general population. In patients with diabetes, the Joint National Committee on the Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) recommends a target BP of <130/80mmHg in order to prevent death and disability associated with high BP.

2.3.1.5 Lipids abnormalities.

Abnormal plasma lipids, characterized by elevated triglycerides and reduced HDL-C, often by elevated apolipoprotein B and non-HDL-C particles are common in patients with established CVDs, T2DM and obesity or metabolic syndrome (Fruchart et al., 2008). In patients with diabetes this pattern of lipids is termed diabetic dyslipidaemia which is one of the modifiable risk factors for T2DM. HDL-C is required for clearance of excess cholesterol from peripheral tissues. However, when HDL-C is reduced, then triglycerides, very low-density lipoprotein (VLDL-C) and LDL-C are all elevated (Daniel et al., 2011). The particle size of LDL-C in patients with diabetes is so small and denser because of elevated triglycerides (Feldman et al., 2018). Elevated triglycerides result from either abnormal overproduction of VLDL-C or impaired lipolysis of triglycerides. Evidence state that patients with T2DM have overproduction of VLDL-C level, which is consequent to elevated free fatty acids, hyperglycaemia, obesity and insulin resistance (Mooradian et al., 2009). several studies confirmed that lowering LDL-C benefits equally both patients with diabetes and without diabetes (Fruchart et al., 2008).

2.3.2 Non-modifiable risk factors.

2.3.2.1 Advanced Age.

Older age is a well-known risk factor for T2DM. Evidence have shown that increased prevalence of T2DM and impaired glucose tolerance increases with aging (Chang et al., 2003). In addition, the prevalence of diabetes is more than two times higher among elderly adults as compared to middle age or young adults and there is high number of incidence diabetic cases (Cowie et al., 2009). Data from the National Health Survey

have shown rapid rise in the incidence of T2DM among elderly population group. The, incidence of diabetes observed in adults aged 65-79 years per 1000 population was 6.0% in 1990, 11.6% in 2000 and 12.4% in 2010 (Corriere et al., 2013). Consequent to high incidence of diabetes in aging populations, it is then projected that number of diabetic cases aged ≥65 years will grow more than 4-fold between 2005 and 2050 (Narayan et al., 2006). There is also evidence which indicates that older individuals develop insulin resistance due to reduced physical activity, obesity and loss of lean body mass, particularly those with a disproportional loss of skeletal muscles (Lee et al., 2017).

2.3.2.2 Ethnicity.

Ethnicity is considered as a non-modifiable risk factor for diabetes with certain ethnic groups being at an increased risk for developing T2DM. An epidemiological study from the U.S. found different prevalence rate of diagnosed diabetes mellitus among adults aged ≥ 20 years old (Spanakis et al., 2013). An increased burden of diabetes was observed in Native Americans (33%) compared to Alaska Natives (5.5%). In addition, similar prevalence rates were observed between the Non-Hispanic whites (7.1%) and Asian Americans (8.4%), whereas Non-Hispanic Blacks and Hispanics Americans had higher prevalence rates of 11.8% and 12.6% respectively (Spanakis et al., 2013).

Another study showed that prevalence of type 2 diabetes ranges from 2% in China to 50% in Pima Indians (Singh et al., 2004). Immigrants in Sweden from the Middle East have 2-3-fold increased risk of T2DM compared to native Swedes. Moreover, these immigrants seem to have a slightly different form of diabetes with early onset and lower C-peptide as compared to Swedish patients. This form of diabetes is also common in patients from Middle East (Glans et al., 2008).

In South Africa Indians have the highest prevalence of diabetes, followed by 8-10% in the Mixed ancestry community (8-10%), 5-8% among blacks and 4% among whites. The large difference in the prevalence of diabetes among ethnic groups exists also due to the influence of genetics and environmental factors. Hence, Indians in South Africa seem to have high genetic predisposition for diabetes susceptibility compared to other ethnic groups. In addition, increased obesity is another factor that contributes to dramatic increase in the T2DM prevalence among ethnic groups.

2.3.2.3 Genetics

An ample body of evidence suggests that there is a genetic component to T2DM risk. The heritability of this disease ranges from 20-80% and evidence is observed from family population and twin-based studies (Meigs et al., 2000);(Poulsen et al., 1999). Several family studies have observed increased risk for T2DM when one or both parents are diagnosed of the disease. For example, study showed that among black South African patients with positive history of diabetes about 82.7% of them had first degree relative with diabetes (Erasmus et al., 2001). In addition, there was a significant maternal aggregation with 64.7% having diabetic mother compared with 27% of those who had diabetic father. This data was corroborated by observed high prevalence of T2DM in patients with diabetic mother (25.4% vs 22.1) and maternal uncles/aunts (31.2% vs 22.2% compared to patients with diabetic father and paternal aunts/uncles respectively in Arabic patients residing in Qatar (Bener et al., 2012).

Similarly, study in Moroccans has shown that familial aggregation of T2DM was prominent and more important in the first-degree relatives than second degree relatives (Benrahma et al., 2011). Earlier evidence has shown that first degree relatives of individuals with T2DM are three times more likely to develop the disease as compared to individuals without positive familial history of diabetes (Florez et al., 2003). Studies have shown that the concordance rate in monozygotic twins is 70% whereas for concordance rate for dizygotic twins has been observed to be 20-30% (Hari Kumar et al., 2014). The familial risk for the disease is strong when studies are restricted to parents aged 35-69 years old, which is also an indication of environmental influence in the disease susceptibility (Almgren et al., 2011).

Moreover, genome wide association studies have shown that single nucleotide polymorphisms (SNPs) of various candidate genes are associated with increased susceptibility for T2DM. These genes include proliferator-activated receptor gamma (PPAR-γ) (Barroso et al., 2006), potassium voltage-gated channel subfamily J member 11 (KCNJ11) (Schwanstecher et al., 2002), hepatocyte nuclear factor 4 alpha (HNF4A) (Hara et al., 2006), transcription factor 7-like 2 (TCF_7L2) (Barroso et al., 2005) and their association with T2DM risk were reproduced in other studies. Furthermore, genome association studies have shown that vitamin D receptor gene polymorphisms are associated with T2DM risk among various population, although the

results are inconclusive. Current study will examine the association of those single nucleotide polymorphisms with T2DM risk.

2.4 Diagnostic criteria for diabetes mellitus.

Diabetes mellitus may be screened or diagnosed based on HbA1c or glucose criteria, either by using the fasting plasma glucose or 2-hr plasma glucose (2-h PG) value after 75-g oral glucose tolerance test (OGTT). A glucose test is performed on patients who are asymptomatic during physical examination or suspicious of either being diabetic or suffering from IFG or IGT. If the results comply with the outlined criteria for diagnosis of diabetes mellitus, then diagnosis is made. Patients can also be subjected to oral glucose tolerance test (OGGT) after an overnight fast (8-12hrs) to detect impaired fasting blood glucose levels, impaired glucose tolerance and diagnose diabetes based on the values depicted on Table 2.1.

Table 2.1: Diagnostic criteria for diabetes, IGT and IFG based on ADA and WHO.

	ADA 2014	**WHO 2016**
Diabetes		
FPG	≥7.0 mmol/l or	≥7.0 mmol/l **or**
2-H plasma glucose	≥11.0 mmol/l	≥11.1 mmol/l
HbA1C	≥6.5%	≥6.5%
IGT		
FPG	Not required	<7.0 **and**
2-h plasma glucose	≥7.8 mmol/l or <11.1mmol	≥7.8 and ≤11.1 mmol/l
IFG		
FPG	5.6-6.9 mmol/l	6.1-6.9 mmol/l **and**
2-h plasma glucose	If measured; ≤11.1 mmol/l	<7.8 mmol/l (**if measured**)

According to (World Health Organization, 2016), participants with fasting plasma glucose levels between (6.1-6.9 mmol/L) are considered to have impaired fasting glucose (IFG) and those with 2-hr plasma glucose levels (≥7.8 and ≤11.1 mmol/L) are considered to have impaired glucose tolerance (IGT). The latter is diagnosed exclusively by using OGGT test. In contrast to ADA, WHO recommend that FPG cut-point for IFG should remain at 6.1 mmol/l the reason being that lowering the cut-point

would increase the proportion of people with IGT who have IFG but decrease the proportion of people with IFG who also have IGT. Participants with either impaired fasting glucose or impaired glucose tolerance are in the prediabetic state. IFG and IGT are the two distinct intermediate states that precedes Type 2DM (Rasmussen et al., 2008). Glycated haemoglobin (HbA1c) greater than 6.5% is also being used to diagnose diabetes.

2.5 Prevalence of type 2 diabetes mellitus (T2DM) in South Africa.

Type 2 diabetes mellitus is the major cause of morbidity and mortality, accounting for over 90% of diabetes cases globally. The number of people with type 2 diabetes mellitus is increasing globally with 80% of diabetes mellitus cases living in low-and middle-income countries (LMICs). In 2014, about 8.5% of adults aged 18 years and older had diabetes. This disease caused 1.6 million deaths in 2014 and high blood sugar was the direct cause of another 2.2 million deaths in the year 2012.

The incidence of this disease varies substantially from one geographical region to the other due to environmental and lifestyle factors. According to WHO, diabetes mellitus will be the seventh leading cause of death in 2030 (Mathers et al., 2006). More recent IDF estimates show that 415 million (uncertainty: 340-536 million) people aged 20-79 years had diabetes in the year 2015 (Ogurtsova *et al.*, 2017). Of that diabetes was accountable for 5.0 million deaths and total health expenditure due to diabetes was estimated at 673 billion US dollar. About a quarter (75%) of diabetes cases aged 20-79 were residing in low-and middle-income countries (LMICs). It is projected that the number of type 2 diabetic cases will rise to 642 million (uncertainty: interval of 521-829 million) in 2040.

Furthermore, (Erasmus *et al.*, 2012) reported a high prevalence of T2DM (28.2%) and 18.1% of undiagnosed diabetes cases within the mixed ancestry population residing in Bellville South, Cape Town, South Africa. These rates are alarming and urgent attention is needed to alleviate the burden of diabetes and to implement preventative methods for early detection of people who are at an increased risk for the disease.

2.6 Vitamin D.

2.6.1 Background.

Vitamin D is a group of fat-soluble vitamins required for the intestinal absorption of calcium and phosphorus. Vitamin D exists in two forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D is required for the maintenance of bone health and body mineral homeostasis. In children, Vitamin D deficiency causes a condition called rickets, which is characterized by poor bone mineralization that leads to soft and weakened bones and bone deformities (Holick et al., 2011);(Sahay et al., 2012). In adults, vitamin D deficiency causes osteomalacia, which is characterized by softened bones, bone pain and muscle weakness (Sahay et al., 2012). The beneficial effect of vitamin D was discovered in 19th century when Sir Edward Mellanby of Great Britain was concerned with extremely high incidence of rickets in the United Kingdom, especially in Scotland.

Sir Edward assumed that rickets could be caused by dietary deficiency. He further went on feeding dogs the Scottish diet, primarily consisting of oatmeal. He kept dogs indoors and deprived them of sunlight (Mellanby et al., 1918). Those dogs developed rickets which was identical to human disease. Interestingly, he managed to cure the disease by feeding dogs cod liver oil and he assumed that cure could be attributed to the effect of vitamin A. McCollum and Davis, discovered vitamin A and they found that it prevented xeropthalmia (McCollum et al., 1913). He proved that vitamin A was not a cure for rickets by heating cod liver oil and he found that cod liver oil still cures rickets and that preparation was no longer able to prevent xeropthalmia and vitamin A deficiency (McCollum et al., 1922). McCollum further concluded that the factor that cured rickets is vitamin D, because vitamin A, vitamin B and vitamin C were already discovered.

The best evidence on the health outcomes of vitamin D to date is documented in bone health. However, accumulating evidence has suggested a role of vitamin D in non-skeletal diseases including cancers, autoimmune disorders, infectious diseases, cardiovascular disease and type 2 diabetes mellitus (Engelman *et al.*, 2010). However, most literature on the non-skeletal health outcomes of vitamin D remains inconclusive.

2.6.2 Sources of vitamin D.

Vitamin D may be obtained through diet or supplementation like other vitamins. Moreover, vitamin D has a unique characteristic that it can also be synthesized from the skin through exposure of the skin to ultra-violet radiation range between 298-315 nm (Spustová et al., 2004)(Holick et al., 2007). Cutaneous synthesis of vitamin D is affected by several factors such as age, skin pigmentation, season, latitude, clothing, use of sunscreens and sun exposure (Chen et al., 2007). Dietary sources of vitamin D are shown on table 2.2 and they includes fish liver oil, cod liver oil, salmon, sardines, tuna, mushrooms and egg yolk (Holick et al., 2007), Many foods are fortified with vitamin D, due to limited amount of vitamin D naturally occurring in food products. The two main forms of vitamin D are derived from different sources: ergocalciferol is derived from the irradiation of ergosterol found in the membranes of yeast and fungus (Bikle et al., 2014) whereas cholecalciferol is derived from conversion of the vitamin D skin precursor 7-dehydrocholesterol (DHRC7) upon exposure to UVB radiation (Holick et al., 2007). Furthermore, a meta-analysis study has found that supplementation with vitamin D_3 is more effective for raising serum 25(OH)D than vitamin D_2 (Tripkovic et al., 2012).

Table 2.2: Dietary sources of vitamin D.

Dietary source	Vitamin D[a] content (IU)
Salmon: Fresh wild	600-1000 Vitamin D_3
Fresh farmed	100-250 Vitamin D_3 or D_2
Sardines canned	300 Vitamin D_3
Tuna canned	236 Vitamin D_3
Mackerel canned	250 Vitamin D_3
Shiitake mushrooms: Fresh	100 Vitamin D_2
Canned	1600 Vitamin D_2
Egg, hard-boiled	20-Vitamin D_2
Supplements: Ergocalciferol	50 000/ Capsule
Cholecalciferol	400, 800, 1000, 2000 etc.
Multivitamin	400, 800 and 1000 Vitamin D_3 or D_2

40 IU is equivalent to 1 ug/l, Adopted from (Holick et al., 2007)

2.6.3 Metabolism of vitamin D.

Vitamin D obtained from both cutaneous synthesis, diet and supplementation is not active, hence it must first undergo series of hydroxylation steps to be converted to the biologically active form. Vitamin D circulates in the blood bound to vitamin D binding protein (VDBP), which is a primary transporter for vitamin D and its metabolites (Wang et al., 2014) . VDBP then transport vitamin D to the liver where it undergoes first hydroxylation to 25-Hydroxyvitamin D or 25(OH)D catalysed by enzyme cytochrome P450 (CYP2R1) enzyme. Serum 25(OH)D is a major circulating metabolite of the vitamin D used to determine patients' vitamin status (DeLuca et al., 2004); (Holick et al., 2007). VDBP further carries hydroxylated vitamin D to the kidneys where it is converted to an active form 1.25-$(OH)_2D$ by enzyme cytochrome P450 (CYP27B1) or 1α-hydroxylase (Zella et al., 2008) However, extra-renal tissues such as dendritic and macrophage cells can convert 25(OH)D to 1.25-$(OH)_2D_3$, due to the presence of CYP27B1 enzyme on their receptors (Van Etten et al., 2005). CYP27B1 is primarily increased by elevated PTH levels in the circulation in response to low calcium levels , (Bouillon et al., 2006) and it is decreased by elevated fibroblast growth factor 23 (FGF23) which regulate phosphate levels in circulation and thus indirectly suppressing production of 1.25-$(OH)_2D_3$ and by direct effects on PTH gland (Bai et al., 2003).1.25-$(OH)_2D_3$ exerts all biological effects of vitamin D (Holick et al., 2009). But, its levels do not correlate with overall vitamin D status, hence is not clinically useful for assessing patients vitamin D status (Holick et al., 2007).

2.6.4 Vitamin D Binding protein (VDBP).

Vitamin D binding protein (VDBP) is a single glycoprotein, member of albumin and α-fetoprotein gene family. This glycoprotein is secreted from the liver and its concentration in healthy individuals ranges from 300-600µmol/l (Blanton et al., 2011), it has a serum short half-life of 2.5-3 days. The total circulating metabolites of vitamin D (about 85-90%) binds with high affinity to the VDBP (Powe et al., 2011), and about 10-15% is bound to albumin whereas about 1% is circulating in free form. VDBP synthesis is oestrogen dependent thus is significantly increased during pregnancy and oestrogen therapy (Heijboer et al., 2012). Also, VDBP levels are significantly reduced in patients with chronic liver disease, kidney disease and reduced in patients with malnutrition (Sinotte et al., 2009).

VDBP exerts other functions such as macrophage activation, binding of fatty acids and clearance of actin filaments due to its actin binding domain (Speeckaert et al., 2006). VDBP is encoded by the group-specific component (Gc) gene, a member of the multigene cluster that includes albumin (ALB) and α-fetoprotein (AFP) genes family, located on chromosome 4q11-q14 (Speeckaert *et al.*, 2006). VDBP has three common alleles (Gc1F, Gc1S and Gc2) and more than 120 rare variants, defined by genetic polymorphisms rs7041 and rs4588 (Braun et al., 1992). Populations of African ancestry have high Gc1F allele frequency Gc1F, whereas Caucasians have markedly high Gc2 allele frequency (Speeckaert *et al.*, 2006). Moreover, certain VDBP variants are associated with low serum 25(OH)D. The Gc1F-1F has the highest affinity to bind 25(OH)D compared to Gc2-2 and is associated with high serum 25(OH)D (Braithwaite et al., 2015).

2.6.5 Parathyroid Hormone (PTH).

Parathyroid hormone (PTH) is a small peptide hormone secreted from parathyroid glands at the back of the neck in response to low calcium levels in the circulation (Lombardi et al., 2011). It plays a vital role in bone mineral homeostasis by regulating and maintaining calcium, phosphorous and activating vitamin D to its active form or 1.25-$(OH)_2D_3$ (Kumar et al., 2011). In state of low calcium levels, PTH stimulates the reabsorption of calcium from the bones and kidneys. PTH serves to increase the activity of CYP27B1 to enhance conversion of 25(OH)D to 1.25-$(OH)_2D_3$ and decrease the activity of 24-Hydroxylase which inhibit production of 1.25-$(OH)_2D_3$ (Christakos et al., 2010). Furthermore, 1.25-$(OH)_2D_3$ may also regulate the activity of calcium sensing receptors (CaSR) which maintains calcium homeostasis through regulation of PTH secretion and renal tubular calcium reabsorption in response to low calcium levels in the circulation (Magno et al., 2011).

CaSR is a G coupled protein receptor (GCPR) expressed primarily on the PTH gland, kidney tissues and is also expressed in other tissues including, thyroid gland, intestine, brain and bones (Ward et al., 2012). PTH synthesis is inhibited by high calcium levels (hypercalcaemia) and high 1.25-$(OH)_2D_3$ in the circulation. Abnormal secretion of PTH is seen in patience with primary hyperparathyroidism, condition that leads to hypercalcaemia (Felsenfeld et al., 2007). Also, high PTH secretion is seen in patients with secondary hyperthyroidism in response to low calcium levels due to vitamin D deficiency or chronic kidney disease (Felsenfeld *et al.*, 2007).

2.6.6 Factors affecting vitamin D levels.

2.6.6.1 Cutaneous synthesis.

Several factors influences cutaneous synthesis of the vitamin D, and these include age, skin pigmentation, season, latitude, clothing, use of sunscreens sun exposure (Chen *et al.*, 2007). Evidence showed that older age is associated with decreased vitamin D synthesis as the skin thickness decreases linearly after the age of 20 years. Cutaneous synthesis of vitamin D in the skin is the function of skin pigmentation and of solar zenith angle which depends on latitude, season and time of the day (Chen *et al.*, 2007). Adequate cutaneous synthesis of vitamin D_3 is met when the skin is exposed to UVB light with photon energy range (290-315nm).

Emerging evidence from observational studies showed that serum 25(OH)D levels differ according to age (Maeda et al., 2013), gender, BMI and season. At latitudes far from the equator during the winter months there is inadequate amount of UVB from the sunlight to allow cutaneous synthesis of 25(OH)D and these observations were confirmed by studies conducted in North America (Holick et al., 1994); (Webb et al., 1988). On the contrary, data from meta-analysis of cross-sectional studies on 25(OH)D globally showed no changes in 25(OH)D levels with latitude after adjusting for age, gender and ethnicity (Hagenau et al., 2009).

The seasonal variation in serum vitamin D among different ethnic groups from different countries is shown in Table 2.3. In addition, evidence on seasonal variation in serum vitamin D levels is thus convincing since majority of the studies reported low serum vitamin D levels in winter (Kull et al., 2009); (Unger et al., 2010); (Martineau et al., 2011), although in some of the warm countries lower levels have been reported in summer due to people avoiding exposure to the sun by staying indoors (Al-Daghri et al., 2012).

Table 2.3: Seasonal variation of vitamin D levels.

Location	Population	25-(OH)D_3 ng/mL		Latitude	References
		Winter	Summer		
Northern Europe	Healthy men and Women	17.6	23.6	59° North	(Kull *et al.*, 2009)
South Africa (Cape Town)	HIV+ and HIV- subjects with/without active TB	12.4	22.8	33° South	(Martineau *et al.*, 2011)
Brazil (Sao Paulo)	University stuff and students	14	34	23°34, South	(Unger *et al.*, 2010)

2.6.6.2 Genetic factors.

Certain genes involved in vitamin D metabolism are thought to influence the variability in the concentrations of 25(OH)D in patients of all population groups (Wang *et al.*, 2010). Emerging evidence from genome wide association studies revealed that single nucleotide polymorphisms (SNPs) of the VDBP affect 25(OH)D concentrations. Variant alleles of other genes such as NADSYN1/DHRC7 that encodes for 7-dehydrocholesterol, were significantly associated with low serum 25(OH)D in Chinese population. Moreover, another study revealed that variant allele Gc and CYP2R1 polymorphisms were significantly associated with low 25(OH)D in Hispanic women and non-Hispanic white (Wang et al., 2014).

2.6.7 Definition and diagnosis of vitamin D deficiency.

Vitamin D deficiency is considered a major public health problem worldwide, as it is associated with increased risk for both skeletal and non-skeletal health outcomes including cancer, autoimmune disorders, infectious diseases, T2DM and CVDs (Engelman *et al.*, 2010). Diagnosis of vitamin D deficiency is based on serum 25(OH)D levels which reflects both vitamin D intake and endogenous production (Holick et al., 2007). Institute of Medicine (IOM) released new guidelines for recommendations of

25(OH)D for dietary reference intakes (DRIs) for calcium and vitamin D, thus updating the 1997 DRIs report (Ross et al., 2011).

IOM stated that as defined by skeletal health, individuals with serum 25(OH)D below 16 ng/ml are at increased risk, whilst those with 25(OH)D levels between (16-20 ng/ml) are potentially at risk and those with 25(OH)D above 20 ng/ml have sufficient vitamin D. IOM concluded that 20 ng/ml of 25(OH)D adequately covered the requirements of at least 97.5% of adult population in relation to bone health. Based on Endocrine Society Practice guidelines, vitamin D deficiency is defined as serum 25(OH)D below 20 ng/ml (Holick et al., 2011). Definition of vitamin D deficiency was based on (i) the elevation of PTH when 25(OH)D levels drops below 20ng/ml, (ii) reduction in PTH levels among elders receiving 800IU vitamin D dose compared to 400IU and (iii) increased intestinal absorption of calcium when 25(OH)D is above 30ng/ml. However, studies have showed that the use of PTH for defining optimal vitamin D status has limitations, due to PTH levels being associated with increase in age, obesity and renal dysfunction. Data from the National Health and Nutrition Examination survey (NHANES) III have showed that change in the diagnosis of vitamin D deficiency from <16ng/ml to <20ng/ml increased prevalence of vitamin D deficiency from 2% to 14% (Saintonge et al., 2009).

2.6.8 Vitamin D receptors (VDR) polymorphisms.

Vitamin D receptor (VDR) is a steroid/thyroid hormone receptor family that functions as a transcriptional activator of many genes. Active vitamin D mediates its biological function on target tissues through binding to VDR (Haussler et al., 2011). Upon binding VDR and after subsequent phosphorylation steps by kinase cascades, the VDR undergoes a conformational change that facilitates its capacity to binding the retinoid X receptor (RXR) thus forming heterodimer, which further interacts with the vitamin D responsive (VDREs) in the promoter region of target genes thus modifying their expression (Haussler *et al.*, 2011); (Zella et al., 2003).

The VDR gene is located on the negative strand of chromosome 12 positioned on the longer arm of the chromosome (12q11.1). VDR gene consists of 14 exons with extensive promoter region capable of generating multiple specific transcripts (Uitterlinden *et al.*, 2004). VDRs are widely distributed in more than 38 tissues, where it controls vital genes involved in bone metabolism, oxidative damages, chronic

diseases and inflammation (Haussler et al., 2008). There are four common single nucleotide polymorphisms (SNPs) of the VDR gene, which are extensively studied namely: *Fok*1 (rs2228570), *Bsm*1 (rs154440), *Apa*1 (rs7975232) and *Taq*1 (rs731236) among other identified polymorphisms (Uitterlinden *et al.*, 2004).

*Fok*1 SNP is located at exon 2 within the 5' end of the VDR gene near the promoter region and *(Bsm*1 and *Apa*1) SNPs are located closely in intron 8 and *Taq*1 SNP at exon 9 at the 3' end of the VDR gene respectively, they are genetically linked (Naito et al., 2007). Among four loci on the VDR gene, the Fok1 SNP is known to affect the structure of VDR protein produced (Wang *et al.*, 2012). This is due to the variant T (f) allele of the Fok1 SNP which encodes 427 amino acid proteins whereas F allele encodes 424 amino acid proteins (Uitterlinden *et al.*, 2004). Consequently, the shorter VDR variant protein seem to function effectively and has a higher binding affinity to 1.25-Dihydroxyvitamin D_3 (Reis et al., 2005).

VDR gene Bsm1, Apa1 and Taq1 SNPs irrespective of their loci on the VDR gene, were reported to have no effect in altering VDR protein structure (Uitterlinden *et al.*, 2004). These SNPs within the VDR gene are associated with altered gene expression or gene function (van Etten et al., 2007), thus the allelic variations in the VDR gene may contribute to genetic predisposition of certain diseases (Palomer et al., 2008). In addition, VDR gene is considered as a particular good candidate gene for susceptibility to T2DM, due to its involvement in the metabolic pathway of T2DM (Nosratabadi et al., 2010).

2.7 Role of vitamin D in the pathogenesis of T2DM

T2DM is a chronic metabolic disease characterized by increased insulin resistance, hyperglycaemia and pancreatic β-cell dysfunction. Studies have shown that physical inactivity, poor nutrition practices and obesity may contribute significantly to the development of T2DM (Moreira et al., 2010). However, the actual aetiopathogenesis of T2DM is unknown. Increasing evidence suggests that vitamin D deficiency may play a role in the pathogenesis of T2DM. This is due to the presence of VDR and VDBP on the pancreatic β-cells (Palomer et al., 2008). Vitamin D deficiency has been shown to alter insulin synthesis and insulin secretion in both human and animal models. However, vitamin supplementation restores insulin secretion and decreases insulin resistance and plasma glucose levels (Palomer et al., 2008);(Moreira et al., 2010).

Additionally, low vitamin D status has been associated with glucose intolerance and occurrence of T2DM in several populations. The proposed mechanisms in which vitamin D deficiency predisposes patients to T2DM may be either through direct action on VDR activation or indirectly via calcium hormones and inflammation (Thorand et al., 2011);(Sung et al., 2012).

Vitamin D is postulated to affect glucose tolerance (Palomer et al., 2008), and reduced concentrations have been associated with pancreatic β-cell dysfunction, insulin receptor down regulation and insulin resistance (Chiu et al., 2004). Evidence state that vitamin D and its metabolites may play a role in preventing T2DM by increasing insulin sensitivity and secretion and overall pancreatic β-cell function (Christakos et al., 2003). There is greatly increased prevalence of T2DM in obesity (Nguyen et al., 2008) and it has been reported that obesity is one of the risk factors for vitamin D deficiency in diverse populations (Iqbal et al., 2017). Obesity is a state of chronic low-grade inflammation and adipose tissues are major sources of inflammation with the infiltration of macrophages as the primary source of cytokines in obese individuals (Bellia et al., 2013) (Xu et at., 2003) (Stienstra et al., 2007). An increase in acute phase proteins such as CRP, pro-inflammatory cytokines and mediators associated with endothelial dysfunction has been reported in T2DM. Vitamin D has anti-inflammatory effects, which reduces chronic inflammation and thus improving insulin sensitivity in patients with obesity low vitamin D levels have been shown to be associated with a rise in CRP and increased proinflammatory cytokines such as IL-6 and TNFα (Bellia et al., 2013), which impairs insulin signalling through different mechanisms, thus leading to increased systemic inflammation, insulin resistance and hyperglycaemia. Moreover, vitamin D supplementation has been shown to reduce inflammatory cytokines such as IL-6 and TNFα which play significant role in inducing insulin resistance (Schleithoff et al., 2006).

2.8. Association studies among vitamin D, vitamin D binding protein and vitamin D receptor polymorphisms.

2.8.1 Association between vitamin D and T2DM.

Previous studies have demonstrated the role of vitamin D deficiency/insufficiency in abnormal glucose metabolism as well as in T2DM (Palomer *et al.*, 2008). Study performed in type 2 diabetes patients residing in the area of Arens and Pireaus in

Greece showed that vitamin D levels were very low in diabetic cases than controls and lower vitamin D correlated negatively with glycosylated haemoglobin levels even after adjustment for confounders (Kostoglou-athanassiou et al., 2013). In addition, vitamin D deficiency has been considered as a possible risk factor for the development of insulin resistance and T2DM by affecting either insulin sensitivity or β-cell function or both (Deleskog et al., 2012) ; (Forouhi et al., 2008).

The proposed mechanisms in which vitamin D deficiency predisposes to T2DM may be either through direct action on vitamin D receptor activation or indirectly via calcium hormones and inflammation (Thorand et al., 2011); (Sung et al., 2012). A recent association study showed that in patients with established T2DM and in the general population, low 25(OH)D levels are associated with higher fasting glucose, insulin resistance and metabolic syndrome (Lips et al., 2016). There is also epidemiological evidence that has shown a positive correlation between circulating 25(OH)D and insulin sensitivity, further demonstrating that vitamin D deficiency may predispose to glucose intolerance, altered insulin secretion and T2DM (Pittas *et al.*, 2010). This data was corroborated by the results of an earlier study which evaluated the association between 25(OH)D and metabolic syndrome (Lu et al., 2009). In this study there was an inverse association of low 25(OH)D with fasting insulin (β=-0.06, p=0.01) and HOMA-IR (β=-0.06, p=0.004) only in overweight subjects.

In a study from Korea, low 25(OH)D was associated with fasting glucose, and OR for diabetes in individuals with serum 25(OH)D < 25, 25-<50 and 50-<75 compared to individuals with serum levels ≥ 75nmol/L were as follows 1.30 (0.91-1.84), 1.40 (0.99-1.98) and 1.73 (1.09-2.74), respectively, p trend <0.001 (Choi et al., 2011). A recent cross-sectional study showed a negative correlation between low serum 25(OH)D with insulin resistance (HOMA-IR: r = -0.200; p=0.03) and fasting plasma glucose (r= -2.95; p= 0.001) but not with BMI (Clemente-Postigo et al., 2015). Furthermore, this data indicates an association of low serum 25(OH)D and diabetes independently of BMI.

On the contrary, the 3rd NHANES data showed that OR for diabetes varied inversely across 25(OH)D quartiles for Non-Hispanic Whites and Mexican Americans (OR 0.25 [CI 0.011-0.60] and 0.17 [0.08-0.37], respectively) for those in the highest quartile compared to the lowest (≥81.0 nmol/L vs ≤43.9nmo/L), respectively, such was not observed in Non-Hispanic Blacks (Scragg et al., 2004). In addition, HOMA-IR was

inversely associated 25(OH)D in Non-Hispanic Whites (p=0.058) and Mexican Americans (p=0.0024) but not in Non-Hispanic Black. It was concluded that lack of inverse association in the Non-Hispanic Black group may reflect decreased sensitivity to vitamin D/or related hormones such as PTH. Another study showed an inverse association of baseline 25(OH)D with T2DM, but the relationship became insignificant after adjusting for BMI (Grimnes et al., 2010). A more recent cross-sectional study in a Bangladeshi population showed that serum vitamin D levels were significantly low in T2DM patients compared to controls (21.30 ± 0.88 vs 43.41 ± 2.52, $p<0.001$), respectively (Rahman et al., 2017). In this study there was a significant negative correlation between serum vitamin D levels and fasting glucose in type 2 diabetes patients ($r = -0.25$, $p < 0.05$).

2.8.2 Association between vitamin D binding protein (VDBP) and T2DM.

Studies demonstrated a positive correlation between serum VDBP concentrations and Active vitamin D (1.25-Dihydroxyvitamin D_3) concentrations (Lauridsen et al., 2005); (Ponda et al., 2014). Moderate association of the VDBP polymorphisms with increased susceptibility to T2DM in Asians have been shown, but not in Caucasians (Wang *et al.*, 2014). Vitamin D binding proteins polymorphisms may predispose to T2DM, moreover VDBP polymorphism 1S and 2 were associated with higher fasting plasma insulin in Japanese subjects (Lips *et al.*, 2016).

2.8.3 Association between vitamin D receptor polymorphisms and T2DM.

VDR is expressed in several tissues including those involved in the regulation of glucose metabolism such as muscles and pancreatic β- cells (Palomer *et al.*, 2008). Moreover, Vitamin D modulates the expression of the insulin receptor genes, insulin secretion, and exert its action on target tissues by binding to the nuclear VDR (Al-Daghri *et al.*, 2012). As vitamin D modulate insulin secretion it is thus possible that genetic variations in the VDR gene may contribute to development of type 2 diabetes mellitus (Palomer *et al.*, 2008). Correlation between VDR polymorphisms and T2DM related metabolic parameters such as glucose intolerance, insulin sensitivity, higher fasting glucose and low vitamin D levels have been reported (Uitterlinden *et al.*, 2004). For example, earlier studies reported that polymorphisms within intron 8 and exon 9 of the VDR affect expression of the protein (Nosratabadi *et al.*, 2010), thus Apa1, Bsm1

and Taq1 are important in the pathogenesis of T2DM through their effects on the VDR gene expression level. These three SNPs (Bsm1, Apa1 and Taq1) are in strong linkage disequilibrium (Uitterlinden *et al.*, 2004).

A study in healthy Bangladeshi Asian population with prevalent vitamin D deficiency showed an association between Apa1 polymorphism (aa) and lower insulin secretion (Hitman et al., 1998). In the Rancho Bernard study, VDR gene Apa1, Bsm1 and Taq1 SNPs were examined and frequency of aa genotype of Apa1 SNP was marginally higher in T2DM patients. Also fasting plasma glucose and prevalence of glucose intolerance was higher in non-diabetic Caucasians with aa genotype compared those with AA genotype and the bb genotype of the Bsm1 SNP was associated with insulin resistance (Oh et al., 2002). Similarly, (Ogunkolade et al., 2002) reported positive association between Bsm1 (bb genotype) and Taq1 (TT genotype) polymorphisms with reduced insulin secretory capacity within the same population. Another study examined association of fasting glucose, low physical activity (PA) and Bsm1 SNP (Ortlepp et al., 2003), they found that males with low PA and carriers of BB genotype of the Bsm1 had elevated fasting glucose. However, such an effect was not observed in those with high PA. Additionally, the VDR gene polymorphisms Bsm1 and Taq1 were associated with an increased susceptibility for T2DM in Saudi Arabian subjects (Al-Daghri *et al.*, 2012)

A cohort study from Brazil reported that the variant genotype ff of Fok1 polymorphism was significantly associated with increased insulin resistance index (HOMA-IR), whilst the variant Ff was significantly associated with increased β-cell function (HOMA-β) and iPTH in patients with the metabolic syndrome (Schuch et al., 2013). However, in patients without the metabolic syndrome the variant genotypes Ff and ff were significantly associated with higher triglycerides levels and lower HDL-C. For Bsm1 only the variant genotype bb was significantly associated with lower 25(OH)D. Of note, a meta-analysis of cross-sectional studies showed that Fok1 polymorphisms were associated with increased risk for T2DM (f vs. F: OR 1.30, 95% CI:1.17-1.45, p< 0.001), especially in East Asians (f vs. F: OR 1.36, 95% CI:1.21-1.54, p< 0.001), while Bsm1 was associated with T1DM (Wang *et al.*, 2012), and no association was observed for Apa1 and Taq1 polymorphisms with either T1DM or T2DM. These findings were corroborated by a study conducted in the Kashmir valley ethnic population group which showed that Fok1 polymorphisms are significantly associated

with increased risk for T2DM and whereas Bsm1 was not (Malik et al., 2017). Also, lower vitamin D deficiency in this population was inversely associated with HbA1c levels.

Additionally, a study in an Emirati population with T2DM showed that allelic variant G of the Fok1 and T of the Bsm1 were associated with increased odds ratios for T2DM {(OR= 1.843, 95%CI=1.288-1.637,p=0.0007, and OR= 1.45,CI=1.032-2.036, p=0.031, respectively)} and consequently, the AG and GG genotypes of the Fok1 and TT genotypes of Bsm1 were associated with increased risk for T2DM {(OR=2.488, 95% CI=1.178-5.256, p=0.015; OR=3.482, 95% CI=1.678-7.224, P=0.0005; OR=1.921, 95% CI=1.009-3,658, P=0.045, respectively)} respectively (Al et al., 2018). More recent study evaluated the association between Taq1 polymorphisms in VDR gene and T2DM in obese Iraq population (Al-darraji et al., 2017). This study showed that variant genotypes TT or Tt of the Taq1 SNP were significantly associated with increased risk for diabetes in patients with T2DM, even after adjusting for HbA1C, BMI, sex, diabetes duration and concurrent dyslipidaemia. Another study which examined Egyptian patients with T2DM with metabolic syndrome showed that variant genotype ff of Fok1 was significantly associated with higher insulin levels, insulin resistance, IL-6 levels, waist circumference and BMI, whereas in those without metabolic syndrome, the variant genotype ff was associated with abnormal lipids profile (elevated TG, TC and LDL-C and lower HDL-C), and Bsm1 SNP was associated with vitamin D deficiency in both groups (Mackawy et al., 2014).

Similarly, in Moroccans patients with prevalent vitamin D deficiency, the variant genotype ff of the Fok1 was associated with reduced risk for development of T2DM (OR=0.35,CI=0.14-0.83 p=0.018) whereas, Bsm1, Apa1 and Taq1 were not (Abdeltif *et al.*, 2014). Moreover, the variant ff of the Fok1 SNP was significantly associated with abnormal lipid (elevated TC, TG and LDL-C and lower HDL-C, $p<0.05$). Only Apa1 polymorphisms was associated with increased systolic blood pressure. Results from another study by (Al-daghri et al., 2014) showed that variant genotype CT and dominant model CT+TT of Bsm1 were significantly associated with increased risk for T2DM (OR=1.7, 95% CI=1.2-2.4, p=0.007; OR=1.5, 95% CI=1.1-2.2, p=0.01, respectively) and further revealed that variant allelic C of Fok1 was significantly associated with reduced risk for development of T2DM (OR=0.73, 95% CI=0.56-0.95, p=0.02). All of the above-mentioned studies are summarized on table 2.4 below.

Table 2.4: Association between VDR polymorphisms and T2DM.

Gene	SNP	SNP genotype	Reported association	Population	Reference
VDR	Fok1 (T/C)				
		FF/GG	Increased β-cell function	Brazilians with MetS	(Schuch et al., 2013)
			increased risk for T2DM	Emirati population with T2DM	(Al et al., 2018)
			Reduced risk for T2DM	Saudi Arabian population	(Al-daghri et al., 2014)
		Ff /AG	Increased risk for T2DM	Emirati population with T2DM	(Al et al., 2018)
		ff/AA	Increased HOMA-IR	Brazilians with MetS.	(Schuch et al., 2013)
			Higher insulin levels, insulin resistance, IL-6 levels, waist circumference and BMI	T2DM Egyptian patients with MetS.	(Mackawy et al., 2014).
			Elevated TG, TC and LDL-C and lower HDL-C	T2DM Egyptian patients without MetS	
			Reduced risk for development of T2DM, elevated TC, TG and LDL-C and lower HDL-C	Morocco patients with prevalent vitamin D deficiency	(Abdeltif *et al.*, 2014)
	Apa1 (T/G)				
		aa/CC	Lower insulin secretion	Bangladeshi Asian with prevalent vitamin D deficiency	(Hitman et al., 1998)
			Higher fasting plasma glucose and prevalence of glucose intolerance	Rancho Bernard study (elderly non-diabetic Caucasian patients)	(Oh et al., 2002).
	Taq1 (T/C)				
			Increased risk for T2DM	Saudi Arabian	
		TT/AA	Increased risk for T2DM	Obese Iraq patients	(Al-darraji et al., 2017)
			Reduced insulin secretory capacity	Elderly non-diabetic Caucasian patients	(Ogunkolade et al., 2002)
		Tt/AG	Increased risk for T2DM	Obese Iraq patients	(Al-darraji et al., 2017)

There is great controversy on the association of VDR polymorphisms and their involvement in the pathogenesis of T2DM. Fok1, Bsm1, Apa1 and Taq1 polymorphisms were not associated with risk for T2DM in Polish population with and without T2DM (Cyganek et al., 2006). Similarly, (Banerjee et al., 2009) showed no association of the Fok1, Bsm1 and Taq1 polymorphisms with increased risk for T2DM in North Indians. Moreover, Apa1 and Taq1 SNPs were not associated with risk for T2DM in Turkish population (Dilmec et al., 2010). The allele f of the Fok1 SNP encodes 427 amino acid proteins whilst the F allele encode 424 amino acid proteins. Studies have reported that shorter VDR variant protein seems to function more effectively and have increased affinity to bind active vitamin D (Reis *et al.*, 2005), subsequently

relatively higher active vitamin D in turn can reduce the risk of developing T2DM by enhancing pancreatic β-cell function and improve insulin resistance (Pittas et al., 2007); (Ozfirat et al., 2010).

Data from various animal and epidemiological based studies have indicated that vitamin D deficiency is a risk factor for the development of T2DM both in animal and humans (Palomer *et al.*, 2008); (Pittas *et al.*, 2010). Various research groups have examined the association of VDR gene polymorphisms (Fok1, Bsm1, Apa1 and Taq1) in relation to increased susceptibility for T2DM in different ethnic groups, however the results are inconsistent. Discrepancies among these reports could be attributed to genetic differences in the populations that were studied or their differential exposure to environmental factors. There is no available data on the association of vitamin D status, vitamin D receptor polymorphisms and T2DM in the mixed ancestral population of Bellville South, Cape Town, South Africa that has been reported to have a high prevalence of diabetes (Erasmus *et al.*, 2012). Therefore, the current study aims to examine the relationship between vitamin D, vitamin D binding proteins and vitamin D receptor polymorphisms (Fok1, Apa1 and Taq1) in diabetic and non-diabetic patients within the mixed ancestral population.

Chapter 3 Purpose of the study

3.1 Research question.

What is the relationship between vitamin D, vitamin D binding proteins and VDR polymorphisms in (T2DM) in the mixed ancestral population of South Africa as investigated in Bellville South, a suburb of Cape Town.

3.2 Problem statement.

T2DM a multifactorial disease caused by interaction of genetics and environmental factors. The condition is characterized by hyper-glycemia and insulin resistance due to β-cell dysfunction or abnormal insulin action. Genetic predisposition of the disease has led to identification of several genes involved in its metabolic pathway. Therefore, it is important to understand the mechanisms underlying the development of the disease and discovery of risk factors which may contribute to its pathogenesis so that early detection of the disease can be made. One of the environmental risk factors for T2DM is vitamin D deficiency as it has been shown to modulate insulin sensitivity and secretion from the pancreatic β-cells. Secondly, presence of the vitamin D receptors on the pancreatic β-cells have suggested the role of vitamin D in glucose regulation. The prevalence of T2DM is very high (28%) within the mixed ancestry population group of Bellville south, Cape Town, South Africa. Additionally, this population has been reported to have high prevalence of obesity and hypertension. However, genetics that predispose this ethnic group to T2DM are poorly understand. Vitamin D receptor gene is considered as a particular good candidate gene for susceptibility to T2DM. Hence, variants of the VDR may possibly play a role in predisposing individuals to T2DM. Association between VDR gene variants and susceptibility has been shown in various ethnic population groups especially of European origin. There is limited data in African populations although the results are often inconclusive. The present study was undertaken to investigate the association between vitamin D, vitamin D binding proteins and VDR polymorphisms in T2DM and non-diabetic patients.

3.3 Hypothesis.

The association between Vitamin D, Vitamin D Binding proteins and Vitamin D Receptor polymorphism in diabetic and non-diabetic patients may contribute and be

associated with the high prevalence of T2DM in the mixed ancestry population of South Africa.

3.4 Aim.

To determine the association between vitamin D, vitamin D Binding proteins and VDR polymorphism in diabetic and non-diabetic patients.

3.4.1 Objectives.

- To assess vitamin D status by measuring serum 25(OH)D levels.
- To measure vitamin binding proteins levels using ELISA kit.
- To determine VDR gene polymorphisms using real-time polymerase chain reaction (RT-PCR).
- To assess the relationship between vitamin D, vitamin D binding proteins and VDR-polymorphisms.

Chapter 4 Methodology.

This chapter focuses on the methods used in the present study. All the methods used are validated and have thus been previously used by other research groups. The analytical methods include determination of the serum vitamin D "25(OH)D" concentrations using a paramagnetic-particle chemiluminescence immunoassay on the Beckman DXI test, serum vitamin D binding protein (VDBP) concentrations determination using Human Vitamin D Binding protein Quantikine ELISA kit and Genomic DNA extraction using salting-out method and genotyping of the VDR single nucleotide polymorphisms using TaqMan SNP Genotyping Assays on real-time PCR platforms.

4.1 Study design.

This is a cross-sectional descriptive study employing quantitative methods. The study aims to investigate the relationship between vitamin D, vitamin D-Binding proteins and VDR polymorphisms in diabetic and non-diabetic subjects.

4.2 Study population.

The current study uses data from the Cape Town Vascular and Metabolic Health (VMH) study, which is an extension of the Cape Town Bellville South Study (Davison et al., 2017) Baseline examinations for the VMH study were conducted between 2014 and 2016. The larger study has received ethics clearance from The Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics, reference number CPUT/HW-REC 2015/H01. The current study comprised of 1603 participants (387 males and 1216 females).

4.3 Inclusion criteria.

- In this study age group criteria were limited to participants aged 20 years or older
- All participants gave informed written consent to participate in the study.

4.4 Exclusion criteria.

4.4.1 Pregnancy.

Pregnancy and consumption of oral contraceptives pills increases the levels of vitamin D binding proteins and vitamin D in the serum (Møller et al., 2012), (Møller et al., 2013). Hence, samples from pregnant women and oral contraceptive pills users were excluded for the current study.

4.4.2 Chronic liver disease

Serum vitamin D binding proteins and vitamin D levels are decreased in patients with chronic liver diseases, due to decreased synthesis of vitamin D binding proteins and albumin from the liver and. Thus, first hydroxylation of vitamin D will be reduced. Hence, samples from patients with chronic liver disease were excluded in the current study.

4.4.3 Renal diseases.

Accumulating data have shown that low serum vitamin D levels are common in patients with chronic kidney diseases at all stages (Chun et al., 2014). In addition, an alteration in the megalin-dependent uptake of DBP in the kidney in CKD would be expected to reduce intracrine conversion of 25(OH)D to active $1.25(OH)_2D_3$. Thus, resulting in complications such as renal osteodystrophy. Hence, samples from patients with renal disease were excluded in the current study.

4.5 Sample size.

The sample size needed for this study has been calculated using the following formula and the estimated prevalence of diabetes within the Bellville South community which was reported as 28% by (Erasmus *et al.*, 2012)

The formulae used is:

$$\mathbf{n = z^2(pq)/e^2}$$

Where:

n = the sample size

z = standard error associated with the chosen level of confidence (1.96)

p = estimated percent in the population

q = 100-p

e = acceptable sample error (5%)

Calculation

$n = z^2(pq)/e^2$

$n = \{1.96^2(28.2 * (100-28.2)\}/5^2$

n = 311

Therefore, sample size needed is 311 participants.

4.6 Ethical considerations

Ethics clearance for the current study was sought from Stellenbosch University Health Research Ethics Committee (HREC), ethics reference number 0719 as well as from the Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences Research Ethics reference number CPUT/HW-REC 2015/H01. Samples for the current study were selected from stored samples from the main study from participants who gave voluntary informed consent. Participants for the main study were informed about their rights and voluntary participation has been explained to them and that they could withdrew from the study at any stage. All information was kept confidential, same as the current study.

4.7. Anthropometric measurements.

4.7.1 weight.

The Omron Body Composition Monitor (BF511: Omron, Japan) was used to measure participants' weight in kilograms (kg). Participants were weighed in light clothing and barefooted. The weight of all participants, except wheelchair bound or those who had impaired posture were measured. The participant would stand on the centre of the flat surface of the scale after it had been zeroed. Hands were placed on the sides and ensuring that subject's weight was evenly distributed, the reading was taken (Tolonen et al., 2002). Readings less than 0.5 kg were rounded off to the nearest lower kilogram while those above 0.5 kg were rounded off to the nearest higher kilogram. The body mass index (BMI) was calculated by dividing the weight and height squared [weight/height^2] (kg/m^2).

4.7.2 Height.

The Omron Body Composition Monitor (BF511: Omron, Japan) was used to measure height of the participants. The stadiometer was used to measure body height to the nearest 0.1 centimetre (cm). The participant had to stand upright on the flat surface of the stadiometer without shoes (Tolonen et al., 2002). The head was placed in the Frankfort plane with hands freely at the sides. The scapular and buttocks were placed close to the vertical sliding metallic bar to ensure accurate readings. The sliding metallic bar gently rested on the subject's head. If the participant was taller than the investigator, then investigator stood on a platform to enable an accurate reading.

4.7.3 Waist circumference.

A non-elastic tape that had been inspected for calibrations and stretch was used to measure the waist circumference (Waist C) (cm). Subjects were asked to stand in an erect position with hands placed on their sides and with their feet and abdominal muscles relaxed. Measurements were taken with the investigator in front of the participant by placing the measuring tape around the natural waist (narrowest part of the torso as seen from the anterior view). For obese participants, the narrowest circumference between the ribs and the iliac crest was measured (Tolonen et al., 2002).

4.7.4 Hip circumference.

The hip circumference (Hip C) (cm) was measured at the maximal circumference over the buttocks. A non-elastic tape was also used for this measurement. The investigator would place the tape around the buttocks on the widest area over the horizontal 23 plane without pressing tightly against the skin, and then take the measurement (Tolonen et al., 2002).

4.7.5 Waist to hip ratio

This was calculated as the waist circumference per hip circumference.

4.8 Laboratory measurements.

4.8.1 Sample collection: whole blood and serum.

4.8.1.1 Whole blood samples.

Whole blood samples were collected into EDTA blood tubes and samples were frozen at -20°C. For the current study, DNA was extracted from these samples and used to determine VDR gene polymorphisms.

4.8.1.2 Serum samples.

Fasting blood samples were collected from participants in BD Vacutainer (SST) tubes (serum), for vitamin D level estimation. Serum samples were centrifuged, and aliquots were frozen at -80°C, which were then used for vitamin D binding protein determination. These results were used to investigate a possible association between Vitamin D, Vitamin D binding proteins and VDR polymorphisms in diabetic and non-diabetic subjects.

4.8.2 Biochemical data collection.

Biochemical measurements: Plasma glucose concentrations were measured using the hexokinase method (Cobas 6000, Roche Diagnostics; Mannheim, Germany), HbA1c was measured using high performance liquid chromatography (HPLC)(Biorad Variant Turbo), Insulin was measured using a Paramagnetic particle assay (Chemiluminescence), Fructosamine was measured using a Colorimetric test nitro-blue-tetrazolium (Roche Cobas c311), LDL-chol (mmol/L) was measured using an Enzymatic Selective Protection –Endpoint assay (Beckman AU), HDL-C (mmol/L) using an Enzymatic Immunoinhibition-Endpoint assay (Beckman AU) and the triglycerides were estimated using a glycerol phosphate oxidase in the presence of peroxidase (GPO-POD) Endpoint assay (Beckman AU).

4.8.3 Definitions and calculations.

4.8.3.1 Body Mass Index (BMI).

Body mass index is widely used as an estimate for prevalence of obesity. Hence, Obesity was classified using the BMI values recommended by (World Health Organization, 2016). The formula used is body weight (kg) / (height (m))^2, was classified as follows:

- Underweight: < 18.50 kg/m^2

- Normal range: 18.50 to 24.99 kg/m^2
- Overweight: ≥ 25.00 to 29.99 kg/m^2
- Obese: ≥ 30.00 kg/m^2

4.8.3.2 Homeostatic model assessment of insulin resistance (HOMA-IR).

HOMA-IR was calculated from fasting insulin and fasting glucose. HOMA-IR had the formula (fasting insulin [mIU/L]) x (fasting glucose[mmol/L])/22.5 (Zhang et al., 2016).

4.8.3.3 Vitamin D status.

Vitamin D deficiency was diagnosed as 25(OH)D levels <20 ng/mL, vitamin D insufficiency was defined as 25(OH)D levels between 20 ng/mL and 29.9 ng/mL, and vitamin sufficiency as 25(OH)D ≥30 ng/mL according to the Endocrine Clinical Society Practice Guidelines for vitamin D deficiency (Holick et al., 2011).

4.8.4 Determination of serum vitamin D levels.

Currently serum 25(OH)D concentrations is the best representative measure of vitamin D status, as serum 1.25-(OH)D_3 is highly regulated by parathyroid hormone (PTH), calcium and phosphorous levels in the circulation and are thus kept within normal ranges (Holick et al., 2008). Therefore, 1.25-(OH)D_3 is not a reliable measure for vitamin D status. In the present study, Vitamin D levels were measured at PathCare Laboratories (Cape Town, Western Cape, South Africa), using the paramagnetic particle chemiluminescence test on a Beckman DXI.

Access 25(OH) vitamin D total assay is a paramagnetic-particle, chemiluminescence Immunoassay used for the quantification of total 25(OH)D concentrations in serum and plasma using Unicell DXI Immunoassay systems.

4.8.5 Determination of serum vitamin D binding proteins (VDBP).

The concentrations of Vitamin D binding protein (VDBP) in serum samples was measured using the Human Vitamin D BP Quantikine ELISA kit (DVDBP0; R&D Systems, Minneapolis, MN, USA) (Freeman et al., 2014). This kit is designed specifically for measurement of DBP in serum, plasma, human milk, saliva and urine. Principle of the assay.

4.8.5.1 Principle of the Human Vitamin D BP Quantikine ELISA.

This kit uses a polyclonal competitive ELISA method. The microwell plates of the kit are pre-coated with antibody specific to DBP. Standards or samples are added along

with biotinylated GC Globulin to microwell plate and will be incubated for the first time. After incubation period, unbound contents are washed away with wash buffer. Streptavidin-Peroxide Conjugate is added, and unbound conjugates are washed away with wash buffer for the second time. TMB substrate is then added to visualize Streptavidin-Peroxidase enzymatic reaction. Streptavidin-Peroxidase catalyse TMB to produce a blue colour product. Addition of acid solution terminate enzyme-substrate reaction thus resulting in yellow colour. Absorbance of standards or samples are measured by ELISA plate reader with a wavelength set at 450 nm. The density of yellow coloration is inversely proportional to the amount of GC globulin captured in the plate.

Intra-Assay CV = 2.2-21%

Inter-Assay CV% = 6.5-2.1%

Reference range =168-367 µg/mL.

Standard deviation (SD) = 51.3 µg/mL.

4.8.5.2 Assay method performance.

All reagents, standards and samples were prepared according to manufacturer's instructions. The assay uses 96-well plate pre-coated with Human Vitamin D BP. Firstly, 50 µl of Assay Diluent RD1-38 was added to each well, with subsequent addition of 50 μl of standard (in duplicate) and sample (not in duplicate) per well. The plate was covered with adhesive strip and incubated for 2 hours at room temperature on a microplate shaker set at 500$\pm$50 rpm. After incubation, fluid was aspirated from each well and washed three times (3x) by filling each well with 400 μl wash buffer using auto washer. In the second incubation, human vitamin D conjugate (200 μl) is added to each well. The plate was then covered with adhesive strip and incubated for 1 hour at room temperature on the microplate shaker. After incubation a wash step was carried again 3 times. Thirdly, 200 μl of substrate solution was added to each well and incubated for 30 minutes at room temperature on the benchtop. After 30 minutes of incubation, stop solution (50 μl) was added to each well, followed by gently taping top of the plate until a colour change appears. Microplate reader set at 450 nm was used to determine the optical density of each well. The density of the colour was proportional to the amount of Vitamin D present in the serum sample.

4.8.6 Determination of the vitamin D receptor polymorphisms.

4.8.6.1 DNA extraction.

Genomic DNA was extracted from the whole blood using a salting-out extraction method (Maurya et al., 2013). 24 tubes were labelled with relevant sample ID. 2 ml of thawed blood were transferred into each tube. Working on ice, 10 ml of lysis buffer were added to each tube. Vortex step was carried out and all tubes were centrifuged at 1500 rpm 4°C for 10 minutes. Supernatant were discarded, and nuclei pellets were retained and resuspended in 10 ml PBS buffer. After resuspension in 10 ml PBS, vortex step was carried out and all tubes were centrifuged at 1500 rpm at 4°C for 10 mins. Supernatant were discarded, and nuclei pellets were dissolved in 3 ml nucleic lysis buffer, 300μl 10% SDS and 30 μl proteinase K (10mg/ml). vortex step was carried out and all tubes were incubated overnight at 55°C (water bath full).

1 ml of saturated 6M NaCl was added to each incubated tube. Vortex step was carried out not too vigorously and the tubes were centrifuged at 2500rpm at 4°C for 30 mins. Supernatants were then transferred into new tubes and pellets were discarded. Two volumes of cold absolute ethanol were added into each tube, and tubes were inverted several times until DNA precipitate appear. DNA was precipitated by adding two volumes of ice cold 100% ethanol (ETOH) and then tube was tilted until DNA precipitates. Precipitated DNA was transferred into Eppendorf tube and resuspended in 1ml of ice cold 70% ethanol. Vortex step was carried out and tubes were centrifuged at maximum speed for 30 minutes. Supernatant were discarded, and pellet retained. Pellets were dissolved in 100-200 µL distilled water. Eppendorf tubes were put on turning apparatus for overnight and DNA concentration was read on Nanodrop Analyzer spectrophotometer (Thermo Scientific™ Nano-Drop™ One Microvolume UV-Vis Spectrophotometers). The 260/280 ratio nm was used to assess purity of the DNA and values between 1.8-2.2 were considered for high quality.

4.8.6.2 Vitamin D receptor polymorphisms genotyping.

The VDR gene polymorphisms were selected to perform genotyping according to the published literature searched on science direct, NCBI, Ensembl.org genome browser 92 and their association with disease susceptibility. The representative SNPs of the VDR selected for genotyping include *Fok1* (*rs2228570*), *Apa1* (rs*7975232*) and *Taq1* (rs*731236*) refer to the table below. Table 4.1 shows the list of SNPs selected for

genotyping based on the literature search on science direct, PubMed, Ensembl.org genome browser 92 and NCBI web, as well as their respective sources. The genomic coordinates for each SNP are given as well as the allele frequencies of underlined alleles of the SNP for African populations and Caucasians of European descendance in the HapMap.

Table 4.1: VDR polymorphisms selected for the present study.

SNP Name and number	sources	Genomic coordinate (Chr12)	Allele	HapMap population	
				CEU	YRI
Fok1 (rs2228570)	(Malik *et al.*, 2017) (Abdeltif *et al.*, 2014)	48272895	C/T/G/A	58.8	80.8
Apa1 (rs7975232)	(Cyganek *et al.*, 2006)	48238837	G/T	42.9	37.2
Taq1 (rs731236)	(Dilmec *et al.*, 2010)	48238757	T/C	56.2	71.2

All SNPs genotyping were performed using the TaqMan SNP Genotyping Assays (Applied Biosystems, USA) (Riedemann et al., 2008) on the QuantStudio 7 Flex system and confirmed by direct sequencing. Primers used for genotyping of the single nucleotide of the VDR are displayed on table 4.2. These primers are pre-designed and found within the TaqMan SNP genotyping Assay.

Table 4.2: Primers used for genotyping from Thermo fisher scientific company.

SNP name	Forward primer 5'-3'	Reverse primer 5'-3'
Fok1(rs2228570)	GGAAGTGCTGGCCGCCATTGCCTCC	TCCCTGTAAGAACAGCAAGCAGGCC
Apa1 (rs7975232)	AAGGCACAGGAGCTCTCAGCTGGGC	CCTCACTGCTCAATCCCACCACCCC
Taq1 (rs731236)	TGGACAGGCGGTCCTGGATGGCCTC	ATCAGCGCGGCGTCCTGCACCCCAG

4.8.6.3 TaqMan SNP Genotyping Assay principle.

This assay uses 5' nuclease to amplify and detect specific SNP allele in a purified genomic DNA sample. The assay consists of sequence-specific primers and two probes each with specific reporter (VIC/FAM) dye attached at the 5' end and a minor groove binding (MGB) with quencher attached at the 3' end. Each TaqMan probe binds to complementary sequence on the DNA template between forward and reverse primer sites. Proximity of the reporter to the quencher results in quenching of the

reporter fluorescence by foster resonance energy transfer (FRET) phenomenon. AmpliTaq Gold DNA polymerase extends primers, and further cleaves the probes hybridized to the target sequence on the DNA template.

Cleavage of the probes separate reporter dye from the quencher, thus resulting in high fluorescence of the reporter. Fluorescence signal generated during PCR amplification when probes hybridize to complementary sequences indicates presence of a SNP allele in the DNA sample. Substantial increase in VIC dye only indicates detection of homozygote Allele 1, increase in FAM dye indicates homozygote Allele 2 and if fluorescence signals both VIC and FAM dyes then it indicates Allele 1-Allele 2 heterozygosity.

4.8.6.4 Method performance for SNP genotyping.

A cocktail mix for one hundred and five (105) samples was first prepared in an Eppendorf tube prior to adding into the MicroAmp fast optical 96-well reaction plate. The cocktail was made from 5 μl TaqMan SNP genotyping Mix, 0.25 μl TaqMan SNP genotyping Assays and 3.75 μl Nuclease free water. Nine microlitres (9 μl) of cocktail mix was added into each well of the plate and subsequently 2 μl genomic DNA was added into the first 93 wells of the plate with respect to sample ID. Two microlitres (2 μl) of nuclease free water were added into the last three wells, thus serving as no template controls (NTC) or negative controls. Each well had total reaction volume of 11 μl (9 μl cocktail + 2 μl genomic DNA/Nuclease free water). The plate was then sealed with MicroAmp optical adhesive film and centrifuged for five seconds (5s) at maximum speed. Thereafter, the plate was loaded in the Quantstudio 7 flexi (Quantum 7, Life Technologies, South Africa) real time PCR systems and PCR was initiated at 95°C for 10 min, followed by 40 cycles of 92°C for 15 sec and 60°C for 60 secs.

A **B**

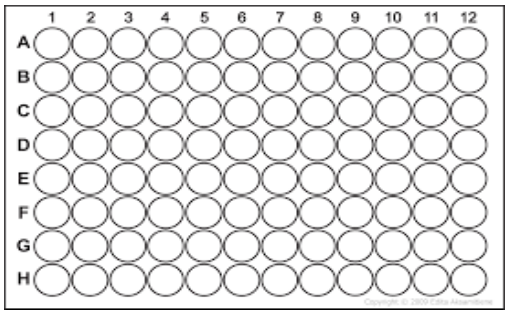

Figure 4.1 Overview of the 96 well plate orientation (A) and real time PCR optical reaction plate (B)

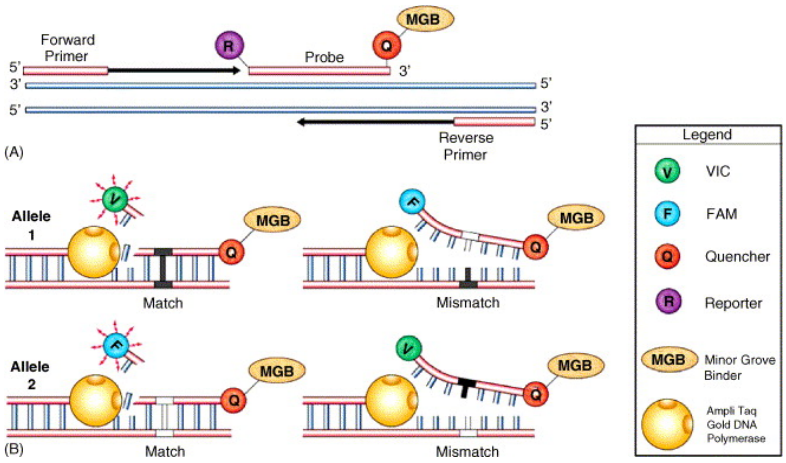

Figure 4.2 Schematic representation of the TaqMan SNP Genotyping assays chemistry overview.

4.9 Statistical analysis

Data were analysed using a software program, Statistica 13.3 (StatSoft, Southern Africa). Comparison analysis between groups were expressed as mean and standard deviation. Correlation studies (R- and P-values) were used to assess the relationship between variables tested. The Pearson Chi-square test was used to determine association between SNPs genotypes and/or allele frequencies and vitamin D deficiency, obesity and hyper-glycaemia categories. A multiple linear regression model (b* and P-value) was used to establish possible association between vitamin D (dependent variable) and other test results. Hardy-Weinberg Equilibrium was tested using the chi-square (x2) method to compare the observed and expected genotypes and alleles frequency between T2DM and non-diabetic patients. For all analysis statistical significance was set at a probability (p) of < 0.05.

Chapter 5 Results

5.1 Characteristics of participants categorized according to gender.

Anthropometric and biochemical characteristics are displayed in table 5.1. The average age of participants was 49.6±15.2 years. Females were significantly older than males (50.2±14.9 (20-90 years) vs 47.8±16.0 range (20-86 years), P=0.0054 respectively). Anthropometric measurements, BMI (kg/m^2), Waist C (cm) and Hip C (cm) were all significantly higher in females than in males (All, P<0.0001). Vitamin D (25(OH)D levels were significantly higher in males compared to females (23.6±7.9 vs 21.5±7.5, P=0.0006) respectively, whereas serum VDBP levels were significantly higher in females compared to males (315.9±76.1 vs 299.1±71.2, P=0.0001) respectively. Females compared to males, they had significantly higher FBG (mmol/L), 2 hr BG (mmol/L), HbA1c) (%), FBI (mIU/L), 2 hr BI (mIU/L), HDL-C (mmol/L), LDL-C (mmol/L), cholesterol (mmol/L) and Parathormone (pmol/L) (All P≤0.0110). In contrast, males compared to females, they had significantly higher Creatinine-S (umol/L), Creatinine-U (mmol/L), Albumin-S (g/L), Sodium-U (mmol/L), Cotinine (ng/mL) and Gamma GT-S (IU/L) were significantly higher in males than in females (All, P≤0.0321).

Table 5.1: Participants characteristics according to gender.

	Total, N= 1603	Males, N= 387	Females, N= 1216	
	Mean ± SD			P-value
Age (years)	49.6±15.2	47.8±16.0	50.2±14.9	**0.0054**
Anthropometric measurements				
BMI (kg/m^2)	29.4±8.1	24.7±6.2	30.9±8.0	**<0.0001**
Waist C (cm)	93.0±17.0	86.4±16.7	95.2±16.5	**<0.0001**
Hip C (cm)	104.9±16.2	94.7±12.3	108.2±15.9	**<0.0001**
WHR	0.89±0.09	0.91±0.09	0.88±0.08	**<0.0001**
Biochemical measurements				
Vitamin D (25(OH)D ng/mL)	22.0±7.6	23.6±7.9	21.5±7.5	**0.0006**
Serum VDBP (µg/mL)	311.8±75.2	299.1±71.2	315.9±76.1	**0.0001**
FBG (mmol/L)	5.84±2.99	5.43±2.24	5.97±3.19	**0.0023**
2 hr BG (mmol/L)	6.68±3.09	5.81±3.06	6.97±3.05	**<0.0001**
HbA1c) (%)	6.25±1.64	5.97±1.30	6.34±1.73	**0.0001**
FBI (mIU/L)	9.30±9.34	7.58±9.48	9.85±9.22	**<0.0001**
2 hr BI (mIU/L)	56.0±53.3	35.0±39.4	62.9±55.5	**<0.0001**
Glucose/Insulin ratio	1.01±0.91	1.30±1.04	0.91±0.84	**<0.0001**
HOMA-IR	2.56±3.47	2.05±3.95	2.73±3.29	**0.0009**
Triglycerides (mmol/L)	1.49±1.32	1.52±1.52	1.49±1.25	0.6762
HDL-C (mmol/L)	1.32±0.36	1.26±0.37	1.34±0.36	**0.0002**
LDL-C (mmol/L)	3.25±1.04	2.96±1.00	3.34±1.04	**<0.0001**
Cholesterol (mmol/L)	5.19±1.19	4.84±1.16	5.30±1.17	**<0.0001**
Cholesterol/HDL ratio	4.13±1.18	4.04±1.26	4.16±1.16	0.0967
Gamma GT-S (IU/L)	46.8±72.4	53.7±97.3	44.6±62.3	0.0321
Creatinine-S (umol/L)	64.7±39.1	77.1±33.3	60.7±40 To conser.0	**<0.0001**
Creatinine-U (mmol/L)	13.5±8.3	15.5±8.7	12.9±8.1	**<0.0001**
ALT (SGPT) (IU/L)	23.6±38.0	26.1±20.7	22.8±42.0	0.1430
AST (SGOT) (IU/L)	26.8±32.3	29.0±16.9	26.1±35.8	0.1229
MDRD (mL/min/1.73m^2)	84.4±12.2	84.5±12.7	84.3±12.0	0.7975
Parathormone (pmol/L)	5.46±3.42	4.92±3.67	5.60±3.34	**0.0110**
Albumin-S (g/L)	42.9±3.3	43.6±3.8	42.7±3.2	**0.0003**
Calcium-S (mmol/L)	2.45±3.14	2.35±	2.48±3.52	0.5906
Phosphate-S (mmol/L)	1.59±15.69	1.04±0.17	1.73±17.64	0.5743
Sodium-U (mmol/L)	108.6±54.0	121.3±57.0	105.3±52.8	**0.0002**
CRP (mg/L)	8.6±15.8	8.5±17.6	8.6±15.1	0.9760
Cotinine (ng/mL)	138.6±159.9	157.7±153.1	132.6±161.6	**0.0078**
Smoking, Yes %(N)	48.3% (758/1568)	59.0% (222/376)	45.0% (536/1192)	**<0.0001***

*Pearson Chi-square

5.2 Stratification of participant characteristics according to gender and glycaemic status (Table 5.2).

Anthropometric measurements including the BMI (kg/m^2), Waist C (cm) and Hip C (cm) were significantly higher in hyper-glycaemic group than in normo-glycaemic males and females (All, P<0.0001). Overall higher BMI (kg/m^2) values were seen in females, which progressively increased in normo-glycaemic (29.7±8.0) to pre-DM (33.2±8.0) and then screen-detected (33.4±7.) and known DM (32.2±7.0) subjects. Vitamin D (25(OH)D ng/mL) was in general significantly decreased in both genders in the hyper-glycaemic sub-groups. In males, Vitamin D (25(OH)D ng/mL) levels were 24.2±8.2 ng/mL in normo-glycaemic and 17.0±6.1 ng/mL in screen-detected DM subjects (P=0.0214) and in females 22.4±7.9 ng/mL in normo-glycaemic and 21.1±6.0 ng/mL in screen-detected DM subjects (P=0.0007). In contrast, there were no significant differences in Serum VDBP (µg/mL) between the glycaemic sub-groups in either male (P=0.5614) or females (P= 0.4813). The glycaemic parameters as expected were significantly increased in the hyper-glycaemic sub-groups in both genders, including FBG (mmol/L), 2 hr BG (mmol/L), HbA1c (%), FBI (mIU/L), 2 hr BI (mIU/L) and HOMA-IR (All, both males and females P<0.0001).

In general, the lipids, including the triglycerides (mmol/L), LDL-C (mmol/L) and Cholesterol (mmol/L) were also significantly increased in both genders in the hyper-glycaemic sub-groups (All, males P≤0.0300, females P<0.0001), while HDL-C (mmol/L) was significantly decreased in both males and females in the hyper-glycaemic sub-groups (All, P≤0.0308). Gamma GT-S (IU/L) was significantly increased in both genders in the hyperglycaemic sub-groups (All, P≤0.0144). Creatinine-S (umol/L) was significantly increased in male subjects with hyper-glycaemia (P =0.0003) and creatinine-U (mmol/L) in female subjects with hyper-glycaemia (P=0.0049). The MDRD (mL/min/1.73m^2) and Sodium-U (mmol/L) were significantly decreased in both genders in subjects with Hyper-glycaemia (All, P≤0.0083). The CRP (mg/L) was near significantly increased in males (P=0.0742) and significantly increased in females (P=0.0031) in the hyper-glycaemic sub-groups. Cotinine (ng/mL) was significantly decreased in both genders in the hyperglycaemic sub-groups (All, P≤0.0024)

Table 5.2: Stratification of participant characteristics according to gender and glycaemic status.

	Males, N=387					Females, N=1216				
	Normo-glycaemic, N=282	**Pre-DM, N=41**	**Screen-detected DM, N=17**	**Known DM, N=47**		**Normo-glycaemic N=757**	**Pre-DM, N=205**	**Screen-detected DM, N=83**	**Known DM, N=171**	
	Mean ± SD				**P-value**	**Mean ± SD**				**P-value**
Age (years)	43.7±15.1	56.5±13.7	59.6±11.8	60.2±12.5	**<0.0001**	46.3±15.1	55.3±13.2	58.2±10.7	57.8±11.3	**<0.0001**
Anthropometric measurements										
BMI (kg/m^2)	23.7±5.9	25.1±6.0	28.7±6.7	28.6±5.9	**<0.0001**	29.7±8.0	33.2±8.0	33.4±7.5	32.2±7.0	**<0.0001**
Waist C (cm)	82.5±14.4	90.1±15.2	100.9±17.4	101.1±19.0	**<0.0001**	91.6±16.7	100.6±14.8	102.7±13.8	100.5±14.7	**<0.0001**
Hip C (cm)	92.9±12.1	96.3±12.9	102.4±12.8	100.9±9.7	**<0.0001**	106.2±16.0	112.3±15.7	112.3±15.0	110.0±14.7	**<0.0001**
WHR	0.89±0.08	0.93±0.07	0.98±0.08	1.00±0.13	**<0.0001**	0.86±0.08	0.90±0.07	0.92±0.07	0.92±0.07	**<0.0001**
Biochemical measurements										
Vitamin D (25(OH)D ng/mL)	24.2±8.2	22.4±6.7	17.0±6.1	24.4±7.1	**0.0214**	22.4±7.9	20.5±7.4	21.1±6.0	19.6±5.5	**0.0007**
Serum VDBP (µg/mL)	302.3±73.7	290.7±58.7	291.7±63.4	290.0±68.6	0.5614	314.7±76.5	319.7±74.3	307.3±74.1	320.7±77.2	0.4813
FBG (mmol/L)	4.66±0.59	5.32±0.69	9.34±4.38	8.82±3.49	**<0.0001**	4.77±0.49	5.34±0.64	8.23±4.21	10.94±5.34	**<0.0001**
2 hr BG (mmol/L)	4.84±1.32	8.55±1.41	15.14±5.69	Not done	**<0.0001**	5.67±1.20	8.83±1.10	14.36±4.99	Not done	**<0.0001**
HbA1c (%)	5.53±0.44	5.71±0.50	7.34±1.62	8.27±2.11	**<0.0001**	5.63±0.47	6.02±0.51	7.65±2.34	9.20±2.43	**<0.0001**
FBI (mIU/L)	6.2±6.5	9.2±12.2	17.1±23.5	11.2±10.7	**<0.0001**	8.2±6.4	12.1±11.6	12.3±8.1	13.2±14.1	**<0.0001**
2 hr BI (mIU/L)	29.7±34.3	66.6±56.7	44.6±29.8	Not done	**<0.0001**	52.2±47.2	100.5±69.4	67.4±45.4	Not done	**<0.0001**
Glucose/Insulin ratio	1.28±0.89	1.35±1.06	0.93±0.61	1.53±1.72	0.2217	0.83±0.49	0.70±0.53	1.02±1.17	1.51±1.60	**<0.0001**
HOMA-IR	1.32±1.60	2.33±3.40	8.21±14.39	3.91±3.23	**<0.0001**	1.77±1.45	2.96±3.20	4.47±3.95	5.85±5.73	**<0.0001**
Triglycerides (mmol/L)	1.35±1.10	1.28±0.70	3.72±4.07	1.92±1.87	**<0.0001**	1.29±0.78	1.77±2.33	1.68±0.85	1.92±1.03	**<0.0001**
HDL-C (mmol/L)	1.28±0.39	1.30±0.35	1.13±0.23	1.14±0.23	**0.0308**	1.36±0.36	1.33±0.35	1.29±0.40	1.28±0.33	**0.0211**
LDL-C (mmol/L)	2.91±1.00	3.13±0.88	3.61±1.15	2.90±0.94	**0.0300**	3.21±1.03	3.55±1.00	3.57±0.94	3.55±1.06	**<0.0001**
Cholesterol (mmol/L)	4.79±1.17	5.03±0.92	5.71±1.47	4.70±1.06	**0.0076**	5.16±1.16	5.53±1.15	5.54±1.09	5.53±1.23	**<0.0001**
Cholesterol/HDL ratio	3.94±1.22	4.09±1.27	5.08±1.45	4.23±1.26	**0.0031**	3.99±1.15	4.37±1.10	4.50±1.15	4.50±1.10	**<0.0001**
Gamma GT-S (IU/L)	47.6±73.2	43.3±33.0	109.1±158.3	79.7±186.5	**0.0144**	41.6±61.3	45.5±46.0	66.8±115.2	46.3±43.1	**0.0059**
Creatinine-S (umol/L)	72.8±15.5	85.8±53.3	85.4±57.9	92.6±61.0	**0.0003**	60.2±47.2	59.0±14.0	60.9±21.1	64.4±32.6	0.5872
Creatinine-U (mmol/L)	14.9±8.4	18.0±9.9	15.6±10.0	16.0±8.7	0.4167	13.0±7.8	13.0±8.9	16.0±9.9	11.1±6.7	**0.0049**
ALT (SGPT) (IU/L)	26.0±21.3	22.6±15.1	29.9±20.5	28.2±21.4	0.5247	21.9±48.8	22.6±16.0	34.8±54.0	21.5±15.1	0.0624
AST (SGOT) (IU/L)	29.2±15.1	27.6±11.1	28.2±16.1	29.4±28.0	0.9490	26.1±42.3	26.3±22.1	32.5±27.4	22.7±14.1	0.2470
MDRD (mL/min/1.73m^2)	86.4±9.2	80.2±17.7	80.9±18.6	78.2±19.4	**<0.0001**	85.4±10.8	84.1±10.7	82.5±14.6	80.9±16.1	**0.0001**
Parathormone (pmol/L)	4.40±2.00	5.76±5.37	6.87±8.51	5.67±4.43	**0.0375**	5.58±3.56	5.41±2.40	5.91±2.33	5.82±3.85	0.6856
Albumin-S (g/L)	43.7±4.1	43.6±3.3	42.5±3.9	43.8±2.7	0.7950	42.8±3.3	42.9±2.7	42.3±2.5	42.1±3.3	0.1341
Calcium-S (mmol/L)	2.35±0.11	2.35±0.11	2.33±0.14	2.34±0.10	0.9553	2.35±0.11	3.02±8.05	2.35±0.09	2.38±0.14	0.2282
Phosphate-S (mmol/L)	1.05±0.16	1.01±0.16	1.05±0.26	1.03±0.18	0.6230	1.10±0.17	4.43±40.52	1.06±0.15	1.11±0.18	0.2331
Sodium-U (mmol/L)	127.0±58.7	128.3±57.2	72.0±34.8	108.4±45.7	**0.0083**	112.4±54.6	99.7±51.9	93.3±47.4	88.3±42.2	**<0.0001**
CRP (mg/L)	7.6±16.5	10.6±20.7	18.7±29.1	8.6±15.6	0.0742	7.3±13.0	10.0±13.4	11.3±14.0	11.0±23.6	**0.0031**
Cotinine (ng/mL)	171.8±152.3	164.3±163.2	115.1±138.2	84.3±133.8	**0.0024**	145.5±162.6	138.2±166.3	74.6±131.3	96.7±153.9	**<0.0001**
Smoking, Yes % (N)	65.8% (179/272)	56.1% (23/41)	41.2% (7/17)	28.3% (13/46)	**<0.0001***	50.5% (374/740)	45.3% (92/203)	25.0% (20/80)	29.6% (50/169)	**<0.0001***

*Pearson Chi-square

Figure 5.1 Summary of the prevalence of optimal Vitamin D (25(OH)D ng/mL) levels in the current population study, categorized according to gender.

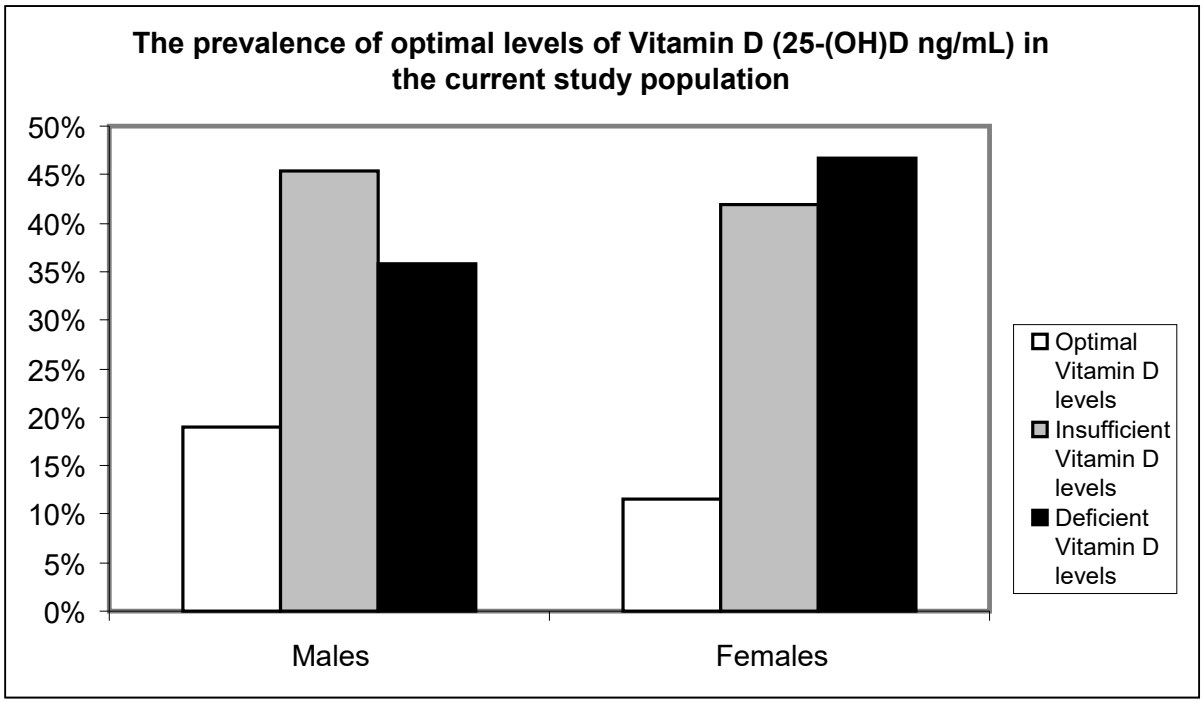

Figure 5.1: *The prevalence of optimal vitamin D levels was very low in the current study population, in males, only 18.9% (38/201) had optimal vitamin D levels, while 45.3% (91/201) had insufficient and 35.8% (72/201) had deficient vitamin D levels. In females, the prevalence of optimal vitamin D levels was also very low, respectively 11.5% (88/767), 41.9% (321/767) and 46.7% (358/767) (Overall, P=0.0033). For the total group, the prevalence was 13.0% (126/968), 42.6% (412/968) and 44.4% (430/968) respectively (data shown elsewhere). Vitamin D (25 OH)D) groupings have been classified as follows: deficient levels are <20 ng/mL, insufficient levels are between 20 and 29 ng/mL and optimal levels are ≥30 to 100 ng/mL (Lakshmi et al., 2015). None of the subjects exceeded the maximum level.*

Figure 5.2 Summary of the prevalence of optimal Vitamin D (25(OH)D ng/mL) based on BMI, categorized according to gender.

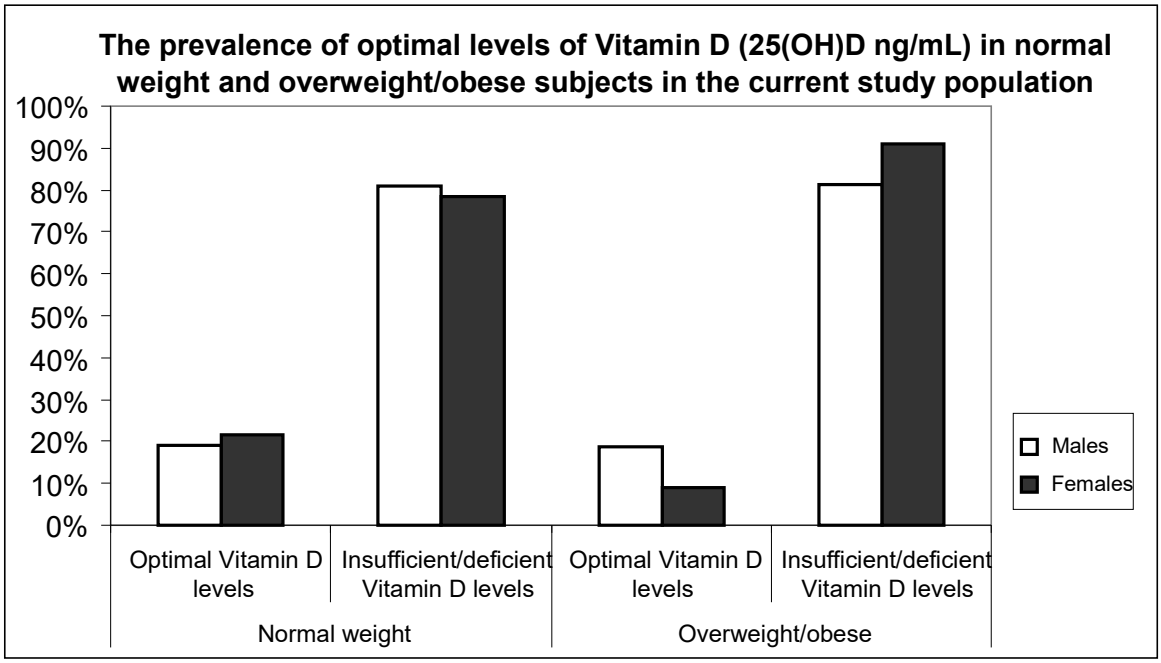

Figure 5.2: *The prevalence of optimal levels of Vitamin D (25(OH)D ng/mL) was very low for both genders in both normal weight and overweight/obese subjects. In males, the prevalence was 19.2% (20/104) in normal weight and 18.8% in overweight/obese subjects (P=0.9310 {Chi-square}). In females, this difference was statistically significant with the prevalence of 21.5% (32/149) and 9.0% (55/614) ((P<0.0001 {Chi-square}) respectively. [The prevalence of obesity has been reported to be high in the community from which the current study population has been recruited (Matsha et al., 2013) and a similarly high prevalence has been shown in the current study population and higher in females than in males (P<0.0001). In the total group 32.8% (524/1596) were of normal weight, 24.1% (384/1596) were overweight and 43.1% (688/1596) were obese. In males, the prevalence was respectively 60.3% (232/385), 24.4% (94/385) and 15.3% (59/385), while in females it was respectively 24.1% (292/1211), 23.9% (290/1211) and 51.9% (629/1211).*

Figure 5.3 Summary of the prevalence of optimal Vitamin D (25(OH)D ng/mL) levels in normo-glycaemic and hyper-glycaemic subjects, categorized by gender.

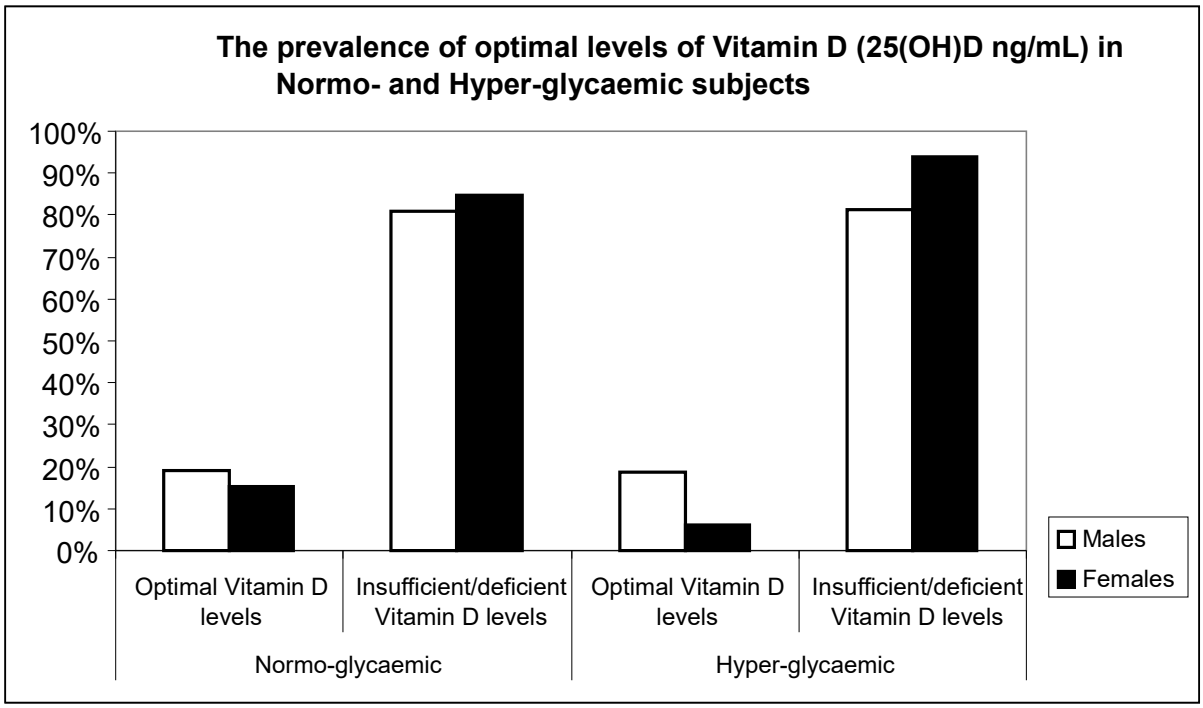

Figure 5.3: *The prevalence of optimal levels of Vitamin D (25(OH)D ng/mL) was very low for both genders in both normo-glycaemic and hyper-glycaemic subjects. In males, the prevalence of optimal levels of Vitamin D (25(OH)D ng/mL) was 18.9% (25/132) in normo-glycaemic and 18.8% (13/69) in hyper-glycaemic subjects, with no significant difference between the glycaemic groups (P=0.9865 {Chi-square}), in females, this difference was statistically significant with the prevalence of 15.1% (69/457) in normo-glycaemic and 6.1% (19/310) in hyper-glycaemic subjects ((P<0.0003 {Chi-square}). [Similar to the prevalence of obesity, the prevalence of hyperglycaemia has been reported to be high in the South African mixed ancestry population (Erasmus et al., 2012).In the current study population, the prevalence of hyperglycaemia was similarly high and also significantly higher in females (P=0.0010). In the total group, 64.8% of subjects (1039/1603) were normo-glycaemic, 15.3% (246/1603) were pre-DM, 6.2% (100/1603) were screen-detected DM and 13.6% (218/1603) were Known DM. In males, the prevalence was respectively, 72.9% (282/387), 10.6% (41/387), 4.4% (17/387) and 12.1% (47/387) and in females respectively 62.3% (757/1216), 16.9% (205/1216), 6.8% (83/1216) and 14.1% (171/1216).*

5.3 Correlation of Vitamin D (25(OH)D ng/mL) levels with anthropometric and biochemical measurements according to gender.

Vitamin D (25-(OH)D ng/mL) showed highly significant correlations in females compared to males with both anthropometric and biochemical measurements. Vitamin D (25(OH)D ng/mL) correlated significantly inversely with the BMI (kg/m^2), the Waist C (cm) and the Hip C (cm) in females, with the strongest inverse correlation with the BMI (kg/m^2) (R=-0.1839, P<0.0001). Vitamin D (25(OH)D ng/mL) correlated significantly positively with serum VDBP (µg/mL) in females (R=0.0969, P=0.0073), but not in males (R=0.0767, P=0.2802). Vitamin D (25(OH)D ng/mL) correlated significantly inversely with the glycaemic measurements, including FBG (mmol/L), 2 hr BG (mmol/L), HbA1c) (%), FBI (mIU/L), 2 hr BI (mIU/L) and HOMA-IR, with the strongest significant inverse correlation with HOMA-IR (R=-0.1907, P<0.0001). In males, Vitamin D (25(OH)D ng/mL) showed a significantly inverse correlation with Post 2 hr BG (mmol/L) (R=-0.2646, P=0.0006).

Similarly, glycaemic profile and Vitamin D (25(OH)D ng/mL) showed highly significant correlations with the lipids in females, but very few in males. In females, Vitamin D (25(OH)D ng/mL) showed significant inverse correlations with triglycerides (mmol/L), LDL-C (mmol/L), Cholesterol (mmol/L), Creatinine-U (mmol/L) and Parathormone (pmol/L) (All, P≤0.0211) and significant positive correlations with HDL-C (mmol/L), Albumin-S and Calcium-S (mmol/L) (P≤0.0008). In males, Vitamin D (25(OH)D ng/mL) showed near-significant weak inverse correlations with the triglycerides (mmol/L), LDL-C (mmol/L) and Cholesterol (mmol/L) (All, P>0.05<0.10) and a significant positive correlation with AST (SGOT) (IU/L) (R=0.1526, P=0.0310). Vitamin D (25(OH) (ng/mL) showed significant inverse correlations with the CRP (mg/L) in males and females, respectively (R= -0.1441, P=0.0413 and R= -0.1049, P=0.0037).

Table 5.3: The correlation of Vitamin D (25(OH)D ng/mL) levels categorized by gender.

	Total, N=968		Males, N=201		Females, N=767	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**
Age (years)	0.0120	0.7092	-0.0230	0.7458	0.0188	0.6032
Anthropometric measurements						
BMI (kg/m^2)	**-0.2036**	**<0.0001**	-0.1111	0.1175	**-0.1839**	**<0.0001**
Waist C (cm)	**-0.1849**	**<0.0001**	-0.1052	0.1383	**-0.1788**	**<0.0001**
Hip C (cm)	**-0.1505**	**<0.0001**	-0.0589	0.4075	**-0.1237**	**0.0006**
WHR	**-0.1204**	**0.0002**	-0.1190	0.0932	**-0.1457**	**0.0001**
Biochemical measurements						
serum VDBP (µg/mL)	**0.0808**	**0.0120**	0.0767	0.2802	**0.0969**	**0.0073**
FBG (mmol/L)	**-0.1503**	**<0.0001**	-0.1270	0.0731	**-0.1494**	**<0.0001**
2 hr BG (mmol/L)	**-0.1790**	**<0.0001**	**-0.2646**	**0.0006**	**-0.1407**	**0.0003**
HbA1c) (%)	**-0.1639**	**<0.0001**	-0.0924	0.1918	**-0.1737**	**<0.0001**
FBI (mIU/L)	**-0.1512**	**<0.0001**	-0.0270	0.7048	**-0.1547**	**<0.0001**
2 hr BI (mIU/L)	**-0.1274**	**0.0003**	-0.1318	0.0896	**-0.1047**	**0.0081**
Glucose/Insulin ratio	**0.0871**	**0.0073**	-0.0007	0.9925	**0.0811**	**0.0258**
HOMA-IR	**-0.1822**	**<0.0001**	-0.0619	0.3860	**-0.1907**	**<0.0001**
Triglycerides (mmol/L)	**-0.1595**	**<0.0001**	-0.1345	0.0570	**-0.1630**	**<0.0001**
HDL-C (mmol/L)	**0.0800**	**0.0129**	0.0039	0.9562	**0.1248**	**0.0005**
LDL-C (mmol/L)	**-0.1695**	**<0.0001**	-0.1385	0.0517	**-0.1619**	**<0.0001**
Cholesterol (mmol/L)	**-0.1526**	**<0.0001**	-0.1339	0.0581	**-0.1385**	**0.0001**
Cholesterol/HDL ratio	**-0.2088**	**<0.0001**	-0.1387	0.0507	**-0.2303**	**<0.0001**
Gamma GT-S (IU/L)	0.0001	0.9980	-0.0410	0.5630	0.0019	0.9577
Creatinine-S (umol/L)	**0.0956**	**0.0029**	0.1222	0.0841	0.0399	0.2713
Creatinine-U (mmol/L)	-0.0626	0.0530	-0.0274	0.7003	**-0.0838**	**0.0211**
ALT (SGPT) (IU/L)	0.0181	0.5734	0.0620	0.3821	-0.0055	0.8797
AST (SGOT) (IU/L)	**0.0903**	**0.0050**	**0.1526**	**0.0310**	0.0599	0.0976
MDRD (mL/min/1.73m^2)	-0.0099	0.7594	-0.0988	0.1662	0.0084	0.8190
Parathormone (pmol/L)	**-0.1696**	**<0.0001**	-0.1236	0.0805	**-0.1658**	**<0.0001**
Albumin-S (g/L)	**0.1321**	**<0.0001**	0.0576	0.4171	**0.1310**	**0.0003**
Calcium-S (mmol/L)	**0.1243**	**0.0001**	0.1360	0.0543	**0.1206**	**0.0008**
Phosphate-S (mmol/L)	0.0363	0.2607	-0.0208	0.7696	0.0708	0.0507
Sodium-U (mmol/L)	0.0291	0.3698	-0.0302	0.6711	0.0326	0.3716
CRP (mg/L)	**-0.1250**	**0.0001**	**-0.1441**	**0.0413**	**-0.1049**	**0.0037**
Cotinine (ng/mL)	**0.0899**	**0.0052**	0.1028	0.1463	**0.0747**	**0.0389**

*Pearson Chi-square

5.4 Correlation of Vitamin D (25(OH)D ng/mL) levels with anthropometric and biochemical measurements categorized by gender and glycaemic status.

Vitamin D (25-(OH)D ng/mL) showed in general significant correlations with both anthropometric and biochemical measurements in normo-glycaemic female subjects and which were of lower significance or absent in the hyper-glycaemic female sub-groups as well as in males. Vitamin D (25(OH)D ng/mL) showed significant inverse correlations with the BMI (kg/m^2), the Waist C (cm) and the Hip C (cm) in normo-glycaemic female subjects (All, P≤0.001) and with the BMI (kg/m^2) in screen-detected DM female subjects (P=0.020). Vitamin D (25(OH)D ng/mL) showed a near-significant positive correlation with serum VDBP (µg/mL) in normo-glycaemic (R=0.087, P=0.064) and a significant positive correlation in pre-DM (R=0.216, P=0.008) female subjects as well as a near-significant positive correlation in screen-detected DM male subjects (R=0.539, P=0.108). Vitamin D (25 OH)D ng/mL) showed in general significant inverse correlations with the glycaemic measures in normo-glycaemic female subjects, including 2 hr BG (mmol/L), HbA1c) (%), FBI (mIU/L), 2 hr BI (mIU/L) and HOMA-IR (All, P≤0.049) as well as significant inverse correlations with FBG (mmol/L) and HbA1c) (%) (All, P≤0.011) in screen-detected DM female subjects, but a significant positive correlation with 2 hr BI (mIU/L) (P=0.022) in screen-detected DM female subjects.

In the males, Vitamin D (25(OH)D ng/mL) showed significant inverse correlations with 2 hr BG (mmol/L) in normo-glycaemic males (P=0.012) and with FBI (mIU/L) in screen-detected males (P=0.010). Vitamin D (25(OH)D ng/mL) showed primarily significant correlations with the lipid profile measurements in the normo-glycaemic, pre-DM and known DM female subjects, but not in the Screen-detected female subjects, these include inverse correlations with the Triglycerides (mmol/L) and LDL-Cl (mmol/L) and positive correlations with HDL-C (mmol/L) (All, P≤0.0490). In the males, in contrast, Vitamin D (25(OH)D ng/mL) correlated significantly inversely with HDL-C (mmol/L) in the pre-DM males (R=-0.390; P=0.044), but near-significantly positively in the screen-detected males (R=0.537; P=0.109). Vitamin D (25(OH)D ng/mL) correlated significantly inversely with parathormone (pmol/L) in normo-glycaemic, pre-DM as well as in screen-detected DM (P≤0.047) female subjects.

Table 5.4: The correlation of Vitamin D (25(OH)D ng/mL) levels with anthropometric and biochemical measurements categorized by gender and glycaemic status.

	Males, N387								Females, N1216							
	Normo-glycaemic, N132		Pre-DM, N27		Screen Detected DM, N11		Known DM, N31		Normo-glycaemic, N457		Pre-DM, N148		Screen-detected DM, N53		Known DM, N109	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Age (years)	-0.035	0.690	-0.198	0.321	0.082	0.811	**0.459**	**0.009**	0.012	0.803	0.141	0.088	0.165	0.239	0.159	0.098
Anthropometric measurements																
BMI (kg/m^2)	-0.126	0.152	0.162	0.421	-0.482	0.133	0.047	0.803	**-0.185**	**<0.001**	-0.120	0.147	**-0.320**	**0.020**	-0.028	0.774
Waist C (cm)	-0.116	0.186	0.101	0.615	-0.555	0.077	0.143	0.442	**-0.186**	**<0.001**	-0.074	0.368	-0.204	0.143	-0.036	0.711
Hip C (cm)	-0.068	0.442	0.213	0.287	-0.382	0.247	-0.037	0.843	**-0.155**	**0.001**	-0.005	0.950	-0.215	0.122	0.017	0.863
WHR	-0.120	0.172	-0.028	0.890	-0.597	0.053	0.164	0.377	**-0.113**	**0.016**	-0.111	0.177	0.119	0.395	-0.180	0.062
Biochemical measurements																
serum VDBP (µg/mL)	0.023	0.791	0.037	0.856	0.539	0.108	0.116	0.535	0.087	0.064	**0.216**	**0.008**	0.084	0.552	0.080	0.410
FBG (mmol/L)	-0.119	0.174	0.243	0.223	-0.278	0.408	-0.243	0.196	-0.090	0.054	0.019	0.816	**-0.351**	**0.010**	-0.144	0.136
2 hr BG (mmol/L)	**-0.222**	**0.012**	0.001	0.995	-0.109	0.749	NA	NA	**-0.121**	**0.010**	-0.148	0.073	-0.187	0.184	NA	NA
HbA1c) (%)	-0.066	0.455	0.088	0.662	-0.018	0.958	-0.354	0.051	**-0.109**	**0.020**	-0.031	0.707	**-0.348**	**0.011**	-0.106	0.272
FBI (mIU/L)	-0.048	0.587	0.310	0.124	**-0.736**	**0.010**	0.025	0.892	**-0.137**	**0.004**	-0.158	0.056	-0.171	0.220	-0.039	0.688
2 hr BI (mIU/L)	-0.122	0.171	0.045	0.825	-0.591	0.056	NA	NA	**-0.094**	**0.049**	-0.153	0.066	**0.322**	**0.022**	NA	NA
Glucose/Insulin ratio	0.029	0.745	-0.310	0.132	**0.707**	**0.022**	-0.106	0.578	**0.121**	**0.010**	**0.162**	**0.049**	0.022	0.880	-0.043	0.658
HOMA-IR	-0.075	0.397	0.301	0.135	-0.600	0.051	-0.080	0.676	**-0.141**	**0.003**	-0.155	0.061	-0.268	0.052	-0.147	0.128
Triglycerides (mmol/L)	-0.134	0.125	-0.112	0.580	-0.436	0.180	0.130	0.486	-0.092	0.050	**-0.235**	**0.004**	0.124	0.378	**-0.323**	**0.001**
HDL-C (mmol/L)	0.064	0.465	**-0.390**	**0.044**	0.537	0.109	-0.052	0.786	**0.132**	**0.005**	0.051	0.541	0.164	0.239	0.116	0.230
LDL-C (mmol/L)	-0.146	0.097	0.171	0.394	0.042	0.907	-0.173	0.361	**-0.092**	**0.049**	**-0.211**	**0.010**	-0.116	0.410	**-0.349**	**<0.001**
Cholesterol (mmol/L)	-0.110	0.208	0.121	0.548	-0.064	0.852	-0.223	0.228	-0.068	0.145	**-0.175**	**0.033**	-0.083	0.555	**-0.315**	**0.001**
Cholesterol/HDL ratio	**-0.212**	**0.015**	0.317	0.107	-0.347	0.327	-0.035	0.853	**-0.183**	**0.000**	-0.198	0.016	-0.198	0.154	**-0.390**	**0.000**
Gamma GT-S (IU/L)	-0.045	0.606	0.021	0.917	0.264	0.433	0.110	0.557	0.052	0.266	-0.056	0.498	0.058	0.678	0.034	0.726
Creatinine-S (umol/L)	0.016	0.859	0.083	0.680	-0.041	0.905	**0.448**	**0.012**	0.018	0.700	0.104	0.208	0.178	0.206	-0.008	0.938
Creatinine-U (mmol/L)	-0.050	0.572	0.160	0.435	0.510	0.109	-0.157	0.399	**-0.125**	**0.008**	-0.067	0.422	0.086	0.546	-0.037	0.706
ALT (SGPT) (IU/L)	0.107	0.224	0.360	0.065	-0.073	0.831	-0.208	0.262	-0.053	0.257	-0.021	0.801	0.241	0.083	0.149	0.121
AST (SGOT) (IU/L)	0.133	0.131	0.468	0.468	0.174	0.610	0.140	0.451	0.021	0.649	-0.022	0.789	0.150	0.285	**0.226**	**0.019**
MDRD (mL/min/1.73m^2)	-0.052	0.557	0.093	0.646	-0.068	0.851	**-0.412**	**0.024**	0.012	0.801	-0.024	0.774	-0.112	0.431	0.009	0.927
Parathormone (pmol/L)	-0.132	0.133	-0.292	0.139	-0.200	0.555	-0.105	0.574	**-0.186**	**<0.001**	**-0.182**	**0.027**	**-0.277**	**0.047**	-0.017	0.864
Albumin-S (g/L)	0.070	0.422	-0.054	0.789	0.041	0.904	0.054	0.775	**0.136**	**0.004**	0.042	0.608	0.173	0.214	**0.193**	**0.045**
Calcium-S (mmol/L)	0.135	0.122	0.109	0.590	0.301	0.368	0.297	0.105	**0.182**	**<0.001**	0.001	0.989	0.098	0.486	0.127	0.189
Phosphate-S (mmol/L)	0.008	0.931	-0.095	0.636	-0.082	0.811	-0.050	0.790	0.068	0.150	0.054	0.520	0.201	0.148	0.056	0.563
Sodium-U (mmol/L)	-0.065	0.461	-0.093	0.653	0.318	0.340	-0.152	0.415	-0.014	0.771	0.050	0.548	-0.187	0.188	**0.227**	**0.021**
CRP (mg/L)	-0.119	0.174	-0.080	0.693	0.145	0.670	-0.146	0.433	-0.081	0.085	-0.113	0.173	-0.134	0.338	-0.012	0.905
Cotinine (ng/mL)	0.097	0.267	-0.064	0.751	-0.063	0.854	0.163	0.381	0.070	0.136	0.144	0.080	0.217	0.119	-0.100	0.303

*Pearson Chi-square

Figure 5.4: Summary of the results of serum VDBP (µg/mL) levels, categorized by gender and vitamin D status.

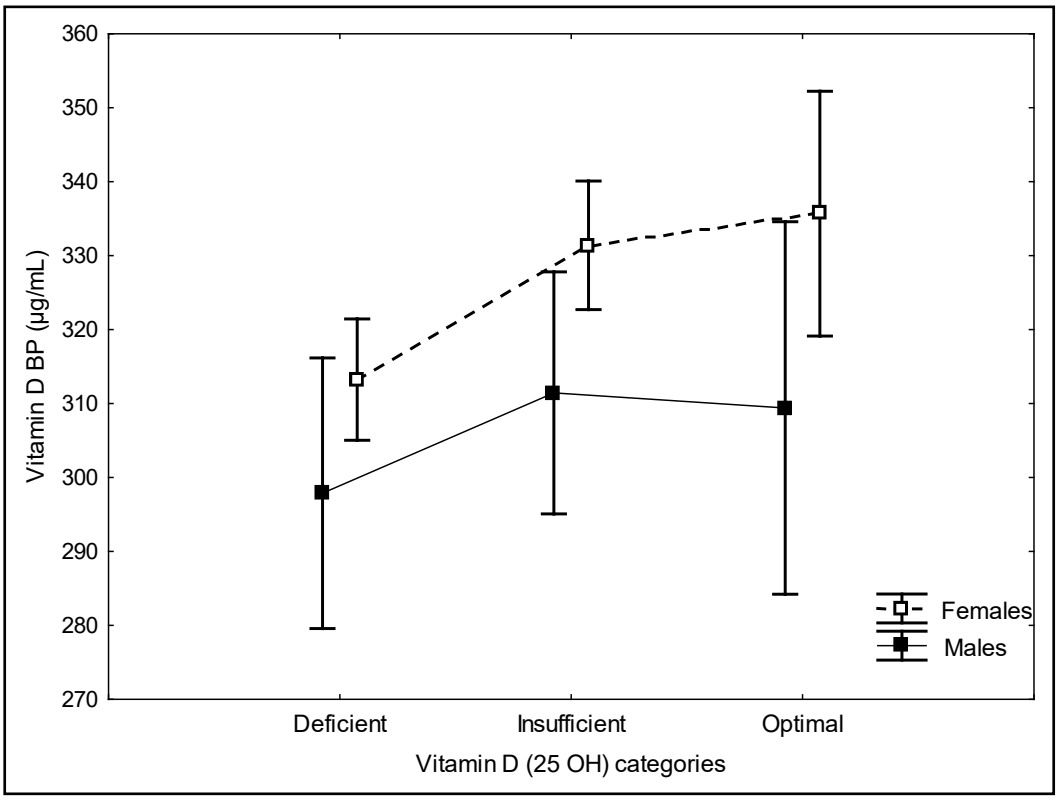

Figure 5. 4: *There were significant differences in the serum VDBP (µg/mL) levels between the genders (P=0.0030) as well as between the Vitamin D (25(OH)D ng/mL) categories, with the Vitamin D (25(OH)D ng/mL) deficient group displaying a marked decrease in Vitamin D BP (µg/mL) levels (P=0.0455), In males, in the Vitamin D optimal category, serum VDBP (µg/mL) was 309.4±80.5 (N38), in the insufficient category 311.4±72.9 (N90) and in the deficient category 297.9±71.3 (N72). In females, the, serum VDBP (µg/mL) was respectively 335.7±71.5 (N88), 331.4±87.8 (N319) and 313.2±75.5 (N358) (µg/mL). [Even though the serum VDBP (µg/mL) was decreased in the Vitamin D deficiency group in both genders, the serum VDBP (µg/mL) showed levels (mean±SD) well within the reference range for normal values in all the Vitamin D categories and for both genders, The reference range for serum VDBP (µg/mL) from healthy volunteers has been reported to be 253± 51.3 (mean±SD) and 168-367 (range) (µg/mL) according to the Human Vitamin D BP Quantikine ELISA kit (DVDBP0; R&D Systems, Minneapolis, MN, USA).*

5.5 The correlation of serum Vitamin D BP (µg/mL) with anthropometric and biochemical measurements categorized by to gender.

There were no significant correlations between serum VDBP (µg/mL)) and any of the anthropometric measurements in either gender. serum VDBP (µg/mL) showed a significant positive correlation with Vitamin D (25(OH)D ng/mL) in females (R=0.0969, P=0.0073), but not in males. Serum VDBP (µg/mL) showed weak near-significant positive correlations with 2 hr BG (mmol/L), HbA1c) (%) and HOMA-IR (All, P>0.05≤0.10) and significant positive correlations with FBI (mIU/L) and 2 hr BI (mIU/L) (All, P≤0.0356), but not in male subjects with any of the glycaemic measurements. Serum VDBP (µg/mL) showed a significant positive correlation with the Triglycerides (mmol/L) in female subjects (P<0.0001) but not in male subjects, while serum VDBP (µg/mL) showed significant positive correlations with LDL -C (mmol/L), Cholesterol (mmol/L) and Gamma GT-S (IU/L) (P≤0.0016) in male subjects, but not in female subjects. serum VDBP (µg/mL) showed a significant positive correlation in females (R=0.0578, P=0.0448) and a near-significant positive correlation in males (R=0.0883, P=0.0836) with Creatinine-S (umol/L). serum VDBP (µg/mL) showed a significant inverse correlation with AST (SGOT) (IU/L) in female subjects (R=-0.0754, P=0.0087). serum VDBP (µg/mL) showed significant positive correlations with the CRP (mg/L) in both males (R=0.1689, P=0.0009) and females (R=0.0831, P=0.0038).

Table 5.5: The correlation of serum Vitamin D BP (µg/mL) with anthropometric and biochemical measurements categorized by gender.

	Total, N=1598		Males, N=385		Females, N=1213	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**
Age (years)	0.0402	0.1082	0.0618	0.2263	0.0264	0.3590
Anthropometric measurements						
BMI (kg/m^2)	-0.0050	0.8435	-0.0184	0.7195	-0.0550	0.0558
Waist (cm)	0.0256	0.3073	0.0430	0.4007	-0.0107	0.7094
Hip (cm)	0.0160	0.5241	0.0212	0.6787	-0.0362	0.2085
WHR	0.0450	0.0725	0.0594	0.2456	0.0557	0.0527
Biochemical measurements						
Vitamin D (25(OH)D ng/mL)	**0.0808**	**0.0120**	0.0767	0.2802	**0.0969**	**0.0073**
FBG (mmol/L)	-0.0025	0.9212	-0.0442	0.3882	0.0005	0.9871
2 hr BG (mmol/L)	0.0513	0.0578	-0.0269	0.6242	0.0561	0.0711
HbA1c) (%)	**0.0537**	**0.0326**	0.0235	0.6476	0.0504	0.0800
FBI (mIU/L)	**0.0672**	**0.0078**	0.0078	0.8791	**0.0610**	**0.0356**
2 hr BI (mIU/L)	**0.0858**	**0.0017**	0.0426	0.4390	**0.0754**	**0.0169**
Glucose/Insulin ratio	**-0.0654**	**0.0106**	-0.0047	0.9289	**-0.0620**	**0.0346**
HOMA-IR	**0.0582**	**0.0214**	-0.0063	0.9029	0.0543	0.0614
Triglycerides (mmol/L)	**0.1208**	**<0.0001**	0.0790	0.1243	**0.1283**	**<0.0001**
HDL-C (mmol/L)	0.0127	0.6117	0.0461	0.3692	-0.0116	0.6855
LDL-C (mmol/L)	**0.0760**	**0.0024**	**0.1607**	**0.0016**	0.0307	0.2863
Cholesterol (mmol/L)	**0.0844**	**0.0007**	**0.1762**	**0.0005**	0.0350	0.2228
Cholesterol/HDL ratio	**0.0661**	**0.0089**	0.0927	0.0732	0.0512	0.0768
Gamma GT-S (IU/L)	**0.0741**	**0.0030**	**0.2182**	**<0.0001**	0.0418	0.1452
Creatinine-S (umol/L)	0.0074	0.7684	0.0883	0.0836	**0.0578**	**0.0448**
Creatinine-U (mmol/L)	**-0.0726**	**0.0249**	-0.0059	0.9346	-0.0700	0.0543
ALT (SGPT) (IU/L)	-0.0394	0.1156	0.0072	0.8876	-0.0402	0.1621
AST (SGOT) (IU/L)	**-0.0774**	**0.0020**	-0.0229	0.6552	**-0.0754**	**0.0087**
MDRD (mL/min/1.73m^2)	**-0.0683**	**0.0069**	-0.0953	0.0641	-0.0535	0.0652
Parathormone (pmol/L)	0.0217	0.4992	0.0092	0.8970	0.0019	0.9581
Albumin-S (g/L)	0.0011	0.9735	0.0637	0.3699	0.0078	0.8296
Calcium-S (mmol/L)	0.0494	0.1211	0.0267	0.7036	0.0591	0.0984
Phosphate-S (mmol/L)	-0.0247	0.4397	0.0832	0.2357	-0.0686	0.0558
Sodium-U (mmol/L)	0.0041	0.9002	0.0921	0.1958	-0.0039	0.9151
CRP (mg/L)	**0.1136**	**<0.0001**	**0.1689**	**0.0009**	**0.0831**	**0.0038**
Cotinine (ng/mL)	-0.0171	0.4981	0.0325	0.5303	-0.0187	0.5196

*Pearson Chi-square

5.6 The correlation of serum Vitamin D BP (µg/mL) with anthropometric and biochemical measurements categorized by gender and glycaemic status.

Serum VDBP (µg/mL) showed very few significant correlations in both genders and all glycaemic sub-groups with both anthropometric and biochemical measurements. Serum VDBP (µg/mL) correlated near-significantly inversely with the BMI (kg/m^2) in normo-glycaemic females (P=0.089) and significantly inversely with the BMI (kg/m^2) (P=0.019), the Waist C (cm) (P=0.027) and near-significantly inversely with the Hip C (cm) (P=0.068) in pre-DM females, while in contrast near-significantly positively with the Waist C (cm) (P=0.101) in normo-glycaemic male subjects. Serum VDBP (µg/mL) showed near-significant positive correlations with 2 hr BG (mmol/L) and 2 hr BI (mIU/L) in normo-glycaemic female subjects (All, P≤0.084) and significant positive correlations with FBI (mIU/L) and 2 hr BI (mIU/L) in screen-detected DM female subjects (All, P≤0.040). In males, serum VDBP (µg/mL) showed a near-significant positive correlation with HbA1c) (%) in normo-glycaemic male subjects (P=0.097) and a significant inverse correlation with 2 hr BG (mmol/L) in pre-DM male subjects (P=0.011).

serum VDBP (µg/mL) showed significant positive correlations with the triglycerides (mmol/L) in Normo-glycaemic and in Pre-DM female subjects (All, P≤0.013). Serum VDBP (µg/mL) showed no correlation with the lipids in any of the glycaemic sub-groups in females, but significant positive correlations with LDL-C (mmol/L) and Cholesterol (mmol/L) in normo-glycaemic males (All, P≤0.002) and a significant positive correlation with HDL-C (mmol/L) (P=0.018) and a near-significant positive correlation with Cholesterol (mmol/L) (P=0.103) in screen-detected DM males. serum VDBP (µg/mL) showed significant positive correlations with Gamma GT-S (IU/L) in normo-glycaemic (R=0.231, P<0.001), screen-detected DM (R=0.706, P=0.0020) and a near-significant positive correlation in known DM (R=0.269, P=0.067) male subjects, but none in the female subjects. serum VDBP (µg/mL) showed significant positive correlations with the CRP (mg/L) in both male (R=0.218, P<0.001) and female (R=0.076, P=0.038) normo-glycaemic subjects.

Table 5.6: The correlation of serum Vitamin D BP (µg/mL) with anthropometric and biochemical measurements categorized by gender and glycaemic status.

	Males, N=387								Females, N=1216							
	Normo-glycaemic N=281		Pre-DM, N=41		Screen detected DM, N=16		Known DM, N=47		Normo-glycaemic N=755		Pre-DM, N=205		Screen-detected DM, N=83		Known DM, N=170	
	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Age (years)	0.110	0.065	-0.059	0.714	-0.166	0.538	0.217	0.143	-0.005	0.900	-0.007	0.925	0.037	0.741	**0.162**	**0.035**
Anthropometric measurements																
BMI (kg/m^2)	0.028	0.639	-0.094	0.565	0.057	0.833	-0.099	0.509	-0.062	0.089	**-0.163**	**0.019**	-0.002	0.984	0.030	0.702
Waist C (cm)	0.098	0.101	-0.020	0.902	0.100	0.713	0.003	0.986	-0.012	0.738	**-0.155**	**0.027**	0.020	0.859	0.090	0.245
Hip C (cm)	0.036	0.548	0.048	0.767	0.126	0.641	0.008	0.957	-0.032	0.385	-0.128	0.068	0.006	0.959	-0.012	0.879
WHR	**0.123**	**0.040**	-0.080	0.621	-0.091	0.736	0.070	0.640	0.028	0.446	-0.006	0.937	-0.054	0.630	**0.244**	**0.001**
Biochemical measurements																
Vitamin D (25(OH)D ng/mL)	0.023	0.791	0.037	0.856	0.539	0.108	0.116	0.535	0.087	0.064	**0.216**	**0.008**	0.084	0.552	0.080	0.410
FBG (mmol/L)	0.010	0.861	-0.111	0.491	0.076	0.778	-0.161	0.286	-0.013	0.724	-0.060	0.390	-0.087	0.434	-0.055	0.480
2 hr BG (mmol/L)	0.015	0.810	**-0.393**	**0.011**	0.082	0.762	NA	NA	0.069	0.059	0.025	0.726	-0.051	0.654	NA	NA
HbA1c) (%)	0.100	0.097	-0.043	0.789	0.069	0.799	-0.016	0.913	0.058	0.114	0.028	0.688	-0.060	0.592	0.028	0.716
FBI (mIU/L)	0.039	0.519	-0.024	0.887	-0.076	0.778	-0.076	0.614	0.036	0.322	0.034	0.636	**0.229**	**0.040**	0.028	0.722
2 hr BI (mIU/L)	0.066	0.278	0.043	0.790	-0.143	0.612	NA	NA	0.064	0.084	0.032	0.654	**0.303**	**0.007**	NA	NA
Glucose/Insulin ratio	-0.030	0.620	0.014	0.934	0.132	0.638	0.086	0.578	-0.047	0.210	-0.034	0.635	**-0.338**	**0.003**	-0.044	0.576
HOMA-IR	0.046	0.451	-0.036	0.827	0.015	0.957	-0.136	0.367	0.037	0.318	0.019	0.792	0.151	0.182	-0.006	0.943
Triglycerides (mmol/L)	0.088	0.146	0.147	0.358	0.203	0.451	0.147	0.326	**0.109**	**0.003**	**0.172**	**0.013**	0.160	0.149	0.100	0.198
HDL-C (mmol/L)	0.052	0.384	-0.195	0.221	**0.600**	**0.018**	0.053	0.725	-0.007	0.845	-0.031	0.657	0.036	0.743	-0.021	0.783
LDL-C (mmol/L)	**0.188**	**0.002**	0.084	0.602	0.262	0.346	0.121	0.422	-0.009	0.812	0.129	0.065	0.125	0.261	0.033	0.670
Cholesterol (mmol/L)	**0.202**	**0.001**	0.006	0.968	0.422	0.103	0.137	0.358	-0.002	0.956	0.120	0.088	0.134	0.226	0.029	0.710
Cholesterol/HDL ratio	0.093	0.125	0.225	0.157	-0.055	0.845	0.063	0.679	0.011	0.766	**0.162**	**0.021**	0.103	0.359	0.026	0.743
Gamma GT-S (IU/L)	**0.231**	**<0.001**	-0.009	0.953	**0.706**	**0.002**	0.269	0.067	0.042	0.252	0.023	0.747	-0.037	0.739	0.106	0.170
Creatinine-S (umol/L)	0.056	0.352	0.013	0.935	0.041	0.879	**0.436**	**0.002**	0.011	0.755	0.135	0.054	0.198	0.074	0.090	0.244
Creatinine-U (mmol/L)	0.007	0.934	-0.318	0.113	**0.699**	**0.024**	-0.026	0.891	**-0.115**	**0.014**	0.087	0.295	-0.254	0.072	-0.020	0.839
ALT (SGPT) (IU/L)	0.010	0.865	0.071	0.658	0.299	0.261	-0.188	0.206	-0.021	0.572	**-0.158**	**0.024**	-0.156	0.158	0.054	0.487
AST (SGOT) (IU/L)	-0.040	0.504	0.010	0.948	0.251	0.349	-0.090	0.547	-0.024	0.516	**-0.150**	**0.032**	**-0.277**	**0.012**	-0.054	0.489
MDRD (mL/min/1.73m^2)	-0.039	0.513	-0.038	0.814	-0.073	0.797	**-0.540**	**<0.001**	0.005	0.900	-0.125	0.076	-0.166	0.136	-0.108	0.165
Parathormone (pmol/L)	0.045	0.610	-0.147	0.464	0.115	0.751	0.034	0.854	-0.006	0.901	-0.048	0.561	0.215	0.119	0.009	0.929
Albumin-S (g/L)	0.055	0.531	0.137	0.497	-0.251	0.485	0.058	0.755	-0.066	0.156	**0.167**	**0.043**	0.141	0.314	0.049	0.616
Calcium-S (mmol/L)	0.057	0.509	-0.017	0.934	0.207	0.565	-0.157	0.391	0.056	0.226	-0.011	0.898	0.136	0.313	0.095	0.325
Phosphate-S (mmol/L)	0.030	0.729	0.002	0.992	0.321	0.365	0.229	0.207	**-0.092**	**0.047**	-0.001	0.990	**-0.266**	**0.046**	0.005	0.956
Sodium-U (mmol/L)	0.068	0.438	0.004	0.985	0.515	0.128	-0.038	0.840	-0.016	0.736	-0.026	0.752	0.058	0.687	0.040	0.690
CRP (mg/L)	**0.218**	**<0.001**	-0.237	0.135	0.024	0.931	0.281	0.056	**0.076**	**0.038**	0.099	0.157	0.045	0.686	0.096	0.214
Cotinine (ng/mL)	-0.038	0.534	0.153	0.341	-0.030	0.913	**0.300**	**0.043**	-0.005	0.884	-0.022	0.751	-0.009	0.936	-0.051	0.512

*Pearson Chi-square

5.7 Hardy-Weinberg Equilibrium.

Hardy-Weinberg equilibrium (HWE) principle states that allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences. In the present study, Fok1, Apa1, Taq1 SNPs were consistent with the Hardy-Weinberg equilibrium (HWE), respectively Chi-squared (X^2), frequency range for the p Allele and for the q Allele for SNP Fok1 (rs2228570): 3.0917, 0.7445 and 0.2555; for SNP Apa1 (rs7975232) respectively 2.9729, 0.6142 and 0.3858;and for SNP Taq1 (rs731236) respectively 0.0157, 0.7174 and 0.2826 [The total number of participants tested was 1603]. The Frequency of variant allele C of the Fok1 SNP was similar in both normo-glycaemic and hyper-glycaemic groups. There was no significant difference in the variant genotypes' distribution between normo-glycaemic and hyper-glycaemic groups (**see Table 5.10**). Also, variant allele A of the Apa1 and T of the Taq1 were similar amongst normo-glycaemic and Hyper-glycaemic groups. The genotype frequencies distribution of these SNPs was similar and not associated with T2DM.

Table 5.7: Hardy-Weinberg Equilibrium testing.

		Genotype	**Observed no**	**Expected no**	**Chi-squared (chi^2)**	**p Allele frequency**	**q Allele frequency**
SNP Fok1 rs2228570	Common Homozygotes	GG	902	888.6			
	Heterozygotes	AG	583	609.8			
	Rare Homozygotes	AA	118	104.6			
					3.0917	**0.7445**	**0.2555**
SNP Apa1 rs7975232	Common Homozygotes	AA	621	604.6			
	Heterozygotes	AC	727	759.7			
	Rare Homozygotes	CC	255	238.6			
					2.9729	**0.6142**	**0.3858**
SNP Taq1 rs731236	Common Homozygotes	AA	824	825.0			
	Heterozygotes	AG	652	650.0			
	Rare Homozygotes	GG	127	128.0			
					0.0157	**0.7174**	**0.2826**

5.8 The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by gender.

As summarized in Table 5.8, there was no significance difference in the genotype or allele frequency distribution of Fok1 (rs2228570), Apa1 (rs7975232) or Taq1 (rs731236) SNPs in both males and females ($P \geq 0.2864$ and $P \geq 0.6347$ respectively).

Table 5.8: The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by gender.

		Total	Males	Females			
	Genotype				**Chi-square***	**df**	**P-value**
SNP Fok1 rs2228570	GG	56.3% (902/1603)	55.3% (214/387)	56.6% (688/1216)			
	AG	36.4% (583/1603)	37.7% (146/387)	35.9% (437/1216)			
	AA	7.4% (118/1603)	7.0% (27/387)	7.5% (91/1216)			
					0.4463	**2**	**0.8000**
	Allele						
	G	74.5% (2387/3206)	74.2% (574/774)	74.5% (1813/2432)			
	A	25.5% (819/3206)	25.8% (200/774)	25.5% (619/2432)			
					0.0000	**1**	**0.9668**
SNP Apa1 rs7975232	AA	38.7% (621/1603)	36.7% (142/387)	39.4% (479/1216)			
	AC	45.4% (727/1603)	47.3% (183/387)	44.7% (544/1216)			
	CC	15.9% (255/1603)	16.0% (62/387)	15.9% (193/1216)			
					0.9770	**2**	**0.6136**
	Allele						
	A	61.4% (1969/3206)	60.3% (467/774)	61.8% (1502/2432)			
	C	38.6% (1237/3206)	39.7% (307/774)	38.2% (930/2432)			
					0.0800	**1**	**0.7719**
SNP Taq1 rs731236	AA	51.4% (824/1603)	53.7% (208/387)	50.7% (616/1216)			
	AG	40.7% (652/1603)	40.1% (155/387)	40.9% (497/1216)			
	GG	7.9% (127/1603)	6.2% (24/387)	8.5% (103/1216)			
					2.5009	**2**	**0.2864**
	Allele						
	A	71.7% (2300/3206)	73.8% (571/774)	71.1% (1729/2432)			
	G	28.3% (906/3206)	26.2% (203/774)	28.9% (703/2432)			
					0.2300	**1**	**0.6347**

*Pearson Chi-square,

*2 x 2 Table, Chi-square

5.9 The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by gender categorized by obesity status.

As summarized in Table 5.9, there was no significance difference in the genotype or allele frequency distribution of Fok1 (rs2228570), Apa1 (rs7975232) or Taq1 (rs731236) SNPs in both normal weight and overweight/obese patients ($P \geq 0.1647$ and $P \geq 0.7471$ respectively).

Table 5.9: The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by gender categorized by obesity status.

		Total	Normal weight	Overweight/obese			
	Genotype				**Chi-square***	**df**	**P-value**
SNP Fok1 rs2228570	GG	56.3% (898/1596)	53.8% (282/524)	57.5% (616/1072)			
	AG	36.3% (580/1596)	39.1% (205/524)	35.0% (375/1072)			
	AA	7.4% (118/1596)	7.1% (37/524)	7.6% (81/1072)			
					2.6087	**2**	**0.2714**
	Allele						
	G	74.4% (2376/3192)	73.4% (769/1048)	75.0% (1607/2144)			
	A	25.6% (816/3192)	26.6% (279/1048)	25.0% (537/2144)			
					0.1000	**1**	**0.7471**
SNP Apa1 rs7975232	AA	38.7% (617/1596)	36.8% (193/524)	39.6% (424/1072)			
	AC	45.4% (724/1596)	48.7% (255/524)	43.8% (469/1072)			
	CC	16.0% (255/1596)	14.5% (76/524)	16.7% (179/1072)			
					3.6076	**2**	**0.1647**
	Allele						
	A	61.3% (1958/3192)	61.2% (641/1048)	61.4% (1317/2144)			
	C	38.7% (1234/3192)	38.8% (407/1048)	38.6% (827/2144)			
					0.0000	**1**	**1.0000**
SNP Taq1 rs731236	AA	51.5% (822/1596)	51.3% (269/524)	51.6% (553/1072)			
	AG	40.5% (647/1596)	41.6% (218/524)	40.0% (429/1072)			
	GG	8.0% (127/1596)	7.1% (37/524)	8.4% (90/1072)			
					1.0099	**2**	**0.6036**
	Allele						
	A	71.8% (2291/3192)	72.1% (756/1048)	71.6% (1535/2144)			
	G	28.2% (901/3192)	27.9% (292/1048)	28.4% (609/2144)			
					0.0000	**1**	**1.0000**

*Pearson Chi-square

*2 x 2 Table, Chi-square.

5.10 The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by glycaemic status.

As summarized in Table 5.10, there was no significance difference in the genotype or allele frequency distribution of Fok1(rs2228570), Apa1 (rs7975232) or Taq1 (rs731236) SNPs in both normo-glycaemic and hyper-glycaemic patients ($P \geq 0.1670$ and $P \geq 0.8711$ respectively).

Table 5.10: The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by glycaemic status.

		Total	Normo-glycaemia	Hyper-glycaemia			
	Genotype				**Chi-square***	**df**	**P-value**
SNP Fok1 rs2228570	GG	56.3% (902/1603)	55.1% (572/1039)	58.5% (330/564)			
	AG	36.4% (583/1603)	37.7% (392/1039)	33.9% (191/564)			
	AA	7.4% (118/1603)	7.2% (75/1039)	7.6% (43/564)			
					2.3586	**2**	**0.3075**
	Allele						
	G	74.5% (2387/3206)	73.9% (1536/2078)	75.4% (851/1128)			
	A	25.5% (819/3206)	26.1% (542/2078)	24.6% (277/1128)			
					0.0300	**1**	**0.8711**
SNP Apa1 rs7975232	AA	38.7% (621/1603)	38.0% (395/1039)	40.1% (226/564)			
	AC	45.4% (727/1603)	47.0% (488/1039)	42.4% (239/564)			
	CC	15.9% (255/1603)	15.0% (156/1039)	17.6% (99/564)			
					3.5790	**2**	**0.1670**
	Allele						
	AA	61.4% (1969/3206)	61.5% (1278/2078)	61.3% (691/1128)			
	CC	38.6% (1237/3206)	38.5% (800/2078)	38.7% (437/1128)			
					0.0000	**1**	**0.9552**
SNP Taq1 rs731236	AA	51.4% (824/1603)	52.2% (542/1039)	50.0% (282/564)			
	AG	40.7% (652/1603)	39.4% (409/1039)	43.1% (243/564)			
	GG	7.9% (127/1603)	8.5% (88/1039)	6.9% (39/564)			
					2.6929	**2**	**0.2602**
	Allele						
	A	71.7% (2300/3206)	71.8% (1493/2078)	71.5% (807/1128)			
	G	28.3% (906/3206)	28.2% (585/2078)	28.5% (321/1128)			
					0.0100	**1**	**0.9107**

*Pearson Chi-square

*2 x 2 Table, Chi-square

5.11 The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by insulin resistance (HOMA-IR) status.

As summarized in Table 5.11, There was no significant difference in the frequency of Fok1 (rs2228570) SNP between subjects with normal insulin resistance and those with moderate/severe insulin resistance, but a near-significant and significant difference in the frequency of Apa1 (rs7975232) and Taq1 (rs731236) SNPs existed between subjects with normal and those with moderate/severe insulin resistance (P=0.0797 and P=0.0360 respectively), but not in their allele frequencies.

Table 5.11: The genotype and allele frequency of (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236) SNPs categorized by insulin resistance (HOMA-IR) status.

		Total	Normal IR	Moderate/severe IR			
	Genotype				**Chi-square***	**df**	**P-value**
SNP Fok1 rs2228570	GG	56.1% (880/1568)	54.8% (653/1191)	60.2% (227/377)			
	AG	36.7% (576/1568)	38.0% (452/1191)	32.9% (124/377)			
	AA	7.1% (112/1568)	7.2% (86/1191)	6.9% (26/377)			
					3.5173	**2**	**0.1723**
	Allele						
	G	74.5% (2336/3136)	73.8% (1758/2382)	76.7% (578/754)			
	A	25.5% (800/3136)	26.2% (624/2382)	23.3% (176/754)			
					0.2400	**1**	**0.6219**
SNP Apa1 rs7975232	AA	38.8% (608/1568)	37.7% (449/1191)	42.2% (159/377)			
	AC	45.5% (713/1568)	47.3% (563/1191)	39.8% (150/377)			
	CC	15.8% (247/1568)	15.0% (179/1191)	18.0% (68/377)			
					6.6505	**2**	**0.0360**
	Allele						
	A	61.5% (1929/3136)	61.3% (1461/2382)	62.1% (468/754)			
	C	38.5% (1207/3136)	38.7% (921/2382)	37.9% (286/754)			
					0.0200	**1**	**0.8845**
SNP Taq1 rs731236	AA	51.3% (804/1568)	52.1% (621/1191)	48.5% (183/377)			
	AG	40.8% (639/1568)	40.7% (485/1191)	40.8% (154/377)			
	GG	8.0% (125/1568)	7.1% (85/1191)	10.6% (40/377)			
					5.0581	**2**	**0.0797**
	Allele						
	A	71.7% (2247/3136)	72.5% (1727/2382)	69.0% (520/754)			
	G	28.3% (889/3136)	27.5% (655/2382)	31.0% (234/754)			
					0.2600	**1**	**0.6100**

*Pearson Chi-square

*2 x 2 Table, Chi-square

5.12 The genotype and allele frequency of SNPs (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236), categorized by Vitamin D (25(OH)D ng/mL) status.

As summarized in Table 5.12**,** there was no significance difference in the genotype or allele frequency distribution of Fok1 (rs2228570), Apa1 (rs7975232) or Taq1 (rs731236) SNPs between subjects with optimal Vitamin D (25(OH)D ng/mL) levels and those with insufficient/deficient levels ($P \geq 0.2036$ and $P \geq 0.6347$ respectively). There was no association between the studied SNPs genotypes/ alleles and vitamin D deficiency.

Table 5.12: The genotype and allele frequency of SNPs (Fok1 rs2228570), Apa1 (rs7975232), and Taq1 (rs731236), categorized by Vitamin D (25(OH)D ng/mL) status.

		Total	Optimal Vitamin D levels	Insufficient/ deficient Vitamin D levels			
	Genotype				**Chi-square***	**df**	**P-value**
SNP Fok1 rs2228570	GG	55.0% (532/968)	54.8% (69/126)	55.0% (463/842)			
	AG	37.0% (358/968)	33.3% (42/126)	37.5% (316/842)			
	AA	8.1% (78/968)	11.9% (15/126)	7.5% (63/842)			
					3.1832	**2**	**0.2036**
	Allele						
	G	73.5% (1422/1936)	71.4% (180/252)	73.8% (1242/1684)			
	A	26.5% (514/1936)	28.6% (72/252)	26.2% (442/1684)			
					0.2300	**1**	**0.6347**
SNP Apa1 rs7975232	AA	38.9% (377/968)	38.9% (49/126)	39.0% (328/842)			
	AC	44.0% (426/968)	42.9% (54/126)	44.2% (372/842)			
	CC	17.0% (165/968)	18.3% (23/126)	16.9% (142/842)			
					0.1679	**2**	**0.9195**
	Allele						
	A	61.0% (1180/1936)	60.3% (152/252)	61.0% (1028/1684)			
	C	39.0% (756/1936)	39.7% (100/252)	39.0% (656/1684)			
					0.0200	**1**	**0.8850**
SNP Taq1 rs731236	AA	50.2% (486/968)	54.0% (68/126)	49.6% (418/842)			
	AG	41.7% (404/968)	38.9% (49/126)	42.2% (355/842)			
	GG	8.1% (78/968)	7.1% (9/126)	8.2% (69/842)			
					0.8400	**2**	**0.6570**
	Allele						
	A	71.1% (1376/1936)	73.4% (185/252)	70.7% (1191/1684)			
	G	28.9% (560/1936)	26.6% (67/252)	29.3% (493/1684)			
					0.1000	**1**	**0.7528**

*Pearson Chi-square

*2 x 2 Table, Chi-square

5.13 The results for serum VDBP(µg/mL) according to SNPs Fok1 (rs2228570), Apa1 (rs7975232) and Taq1 (rs731236) genotypes.

As summarized below in Table 5.13, there was no significance differences in the serum VDBP levels as evaluated against SNPs Fok1 (rs2228570), Apa1 (rs7975232) and Taq1 (rs731236 genotypes). These results demonstrate no association between SNPs genotypes and serum VDBP levels.

Table 5.13: Vitamin D binding protein (µg/mL) according to SNPs Fok1 (rs2228570), Apa1 (rs7975232) and Taq1 (rs731236) genotypes.

		Vitamin D binding protein (µg/mL)			
		Mean ± SD			**P-value**
SNP Fok1 rs2228570	**Genotype**	GG	AG	AA	
		310.4±76.1	313.5±74.2	314.6±74.5	0.6787
		Mean ± SD			**P-value**
SNP Apa1 rs7975232	**Genotype**	AA	AC	CC	
		310.0±71.9	313.1±79.5	312.8±71.0	0.7392
		Mean ± SD			**P-value**
SNP Taq1 rs731236	**Genotype**	AA	AG	GG	
		311.3±74.5	312.8±76.4	310.4±74.7	0.9009

5.14 Multiple Linear regression analysis for dependent variable Vitamin D (25(OH)D ng/mL).

Multiple linear regression analysis revealed that Age, serum VDBP levels and creatinine were predictors of vitamin D levels in females of the total population group and the hyper-glycaemic female group. In hyper-glycaemic females, only triglycerides remained predictors of vitamin D levels. In the total group LDL-C was a predictor of vitamin D levels in both males and females, but in the glycaemic group statistical significance was observed in the hyper-glycaemic females not males. PTH levels were predictors of vitamin D levels in both males and glycaemic groups except in normo-glycaemic male group. All other variables did not reach statistical significance in either males and females of the total group and the glycaemic sub-groups. In normo-glycaemic males 19.5% variation in 25(OH)D was attributed by increased LDL-C and in the hyper-glycaemic group 15.5% it was attributed by PTH and CRP. In normo-glycaemic 12.8% variation in 25(OH)D was attributed by LDL-C, serum Creatinine and PTH, whereas in hyper-glycaemic group 16.1% was attributed by increased age, serum VDBP, triglycerides, LDL-C, creatinine and PTH. Results of the regression analysis are summarised below on table 5.13.

Table 5.14: Multiple linear regression analysis for dependent variable Vitamin D (25-(OH)D ng/mL).

	Total, N=968		Males, N=201		Females, N=767		Normo-glycaemic males		Hyper-glycaemic males		Normo-glycaemic females		Hyper-glycaemic females	
	b*	P-value	b*	P-value	b*	P-value	b*	P-value	b*	P-value	b*	P-value	b*	P-value
Adjusted R^2	0.149 (14.9%)		0.175 (17.5%)		0.140 (14.0%)		0.195 (19.5%)		0.155 (15.5%)		0.128 (12.8%)		0.161 (16.1%)	
Age (years)	0.072	**0.048**	-0.037	0.675	0.105	**0.014**	-0.036	0.763	0.250	0.192	0.092	0.143	0.160	**0.010**
BMI (kg/m^2)	-0.033	0.380	0.013	0.875	-0.065	0.123	-0.111	0.310	0.362	0.072	-0.038	0.509	-0.050	0.432
Serum VDBP (µg/mL)	0.090	**0.004**	0.031	0.665	0.106	**0.003**	0.002	0.979	0.226	0.136	0.070	0.144	0.155	**0.007**
FBG (mmol/L)	-0.060	0.068	-0.032	0.673	-0.067	0.071	-0.082	0.413	-0.083	0.588	-0.074	0.147	-0.023	0.702
FBI (mIU/L)	-0.061	0.074	-0.046	0.573	-0.064	0.099	0.103	0.353	-0.108	0.505	-0.018	0.740	-0.064	0.290
Triglycerides (mmol/L)	-0.105	0.078	-0.102	0.251	-0.097	0.188	-0.141	0.150	-0.223	0.286	0.027	0.631	-0.363	**0.016**
HDL-C (mmol/L)	0.036	0.307	-0.088	0.252	0.056	0.176	-0.082	0.394	-0.308	0.051	0.069	0.218	0.027	0.682
LDL-C (mmol/L)	-0.169	**<0.001**	-0.236	**0.004**	-0.161	**<0.001**	-0.233	**0.033**	-0.181	0.224	-0.164	**0.003**	-0.169	**0.006**
Gamma GT-S (IU/L)	0.060	0.132	0.282	0.008	-0.015	0.745	0.232	0.101	0.153	0.387	-0.016	0.822	0.054	0.420
Creatinine-S (umol/L)	0.251	**<0.001**	0.202	0.237	0.350	**<0.001**	-0.078	0.702	0.701	0.104	0.414	**<0.001**	0.300	**0.016**
Creatinine-U (mmol/L)	-0.047	0.134	-0.022	0.754	-0.059	0.099	-0.033	0.709	0.173	0.246	-0.050	0.289	-0.055	0.337
ALT (SGPT) (IU/L)	0.019	0.708	-0.057	0.667	0.079	0.180	-0.167	0.317	0.127	0.622	-0.012	0.882	0.145	0.118
AST (SGOT) (IU/L)	0.040	0.473	-0.025	0.876	0.021	0.725	0.136	0.517	-0.191	0.448	0.050	0.567	0.024	0.798
MDRD ($mL/min/1.73m^2$)	0.066	0.255	-0.172	0.246	0.208	0.006	-0.327	0.098	0.003	0.994	0.198	0.051	0.252	0.033
Parathormone (pmol/L)	-0.275	**<0.001**	-0.280	**0.015**	-0.280	**<0.001**	-0.155	0.104	-0.819	**0.024**	-0.326	**<0.001**	-0.240	**0.001**
Albumin-S (g/L)	0.069	0.046	0.041	0.695	0.057	0.138	0.074	0.599	-0.107	0.548	0.017	0.766	0.038	0.548
Calcium-S (mmol/L)	0.031	0.314	0.155	0.122	0.036	0.296	0.208	0.092	-0.177	0.471	0.144	0.010	0.067	0.218
Phosphate-S (mmol/L)	-0.007	0.905	-0.106	0.174	-0.018	0.793	-0.077	0.404	-0.167	0.317	-0.047	0.334	0.154	0.288
Sodium-U (mmol/L)	-0.046	0.163	-0.092	0.244	-0.029	0.437	-0.076	0.438	-0.103	0.580	-0.026	0.594	-0.047	0.413
CRP (mg/L)	-0.066	0.050	-0.150	0.069	-0.030	0.418	-0.167	0.142	-0.513	**0.010**	-0.022	0.661	-0.069	0.264
Cotinine (ng/mL)	0.019	0.566	0.057	0.486	0.015	0.700	-0.035	0.727	0.089	0.580	0.013	0.791	0.038	0.521

Chapter 6 Discussion

Diabetes mellitus has reached epidemic proportions worldwide and is a major public health problem. This was a cross-sectional study which was designed to investigate the association between 25(OH)D, vitamin D binding proteins and VDR (Fok1, Apa1 and Taq1) polymorphisms in T2DM patients in the mixed ancestry population group residing in Bellville South, Cape Town, South Africa. This population is characterized by a high prevalence of T2DM (28%) as previously reported by (Erasmus *et al.*, 2012) , hypertension and obesity (Matsha *et al.*, 2013). T2DM is a multifactorial disease caused by a complex interaction of genetics and environmental factors (Palomer *et al.*, 2008). In the present study vitamin D deficiency (44%) and insufficiency (42.6%) were highly prevalent in the population studied with optimal 25(OH)D levels being observed in only 13% of subjects. The overall vitamin D status of the whole population group was insufficient (22.0±7.6 ng/mL).

It has been reported that people living in Southern Hemispheres in cities like Cape Town and Buenos Aires produce far less vitamin D during the winter months and this has been corroborated by the observation of a high prevalence of vitamin D deficiency (62.7%) in black Africans residing in Cape Town, South Africa (Martineau *et al.*, 2011). A high prevalence of vitamin D deficiency (40%) has also been observed in Moroccan (Abdeltif *et al.*, 2014) and in Caribbean (42%) subjects with T2DM (Vélayoudom-céphiseet al., 2011).

In the present study, 25(OH)D levels and VDBP varied according to gender with males having higher 25(OH)D levels (P=0.0006) and females with significantly higher serum VDBP levels (P<0.0001). In accordance with our observations , (Blanton *et al.*, 2011) observed higher serum VDBP levels in healthy females compared to male subjects and Another study from Belgium reported 10% higher serum VDBP levels in females than males (Bolland et al, 2007). In the present study the vitamin D deficient groups in both genders had low serum VDBP levels compared to the optimal vitamin D groups. Even though serum VDBP levels were lower in the vitamin D deficient group, they were still within the normal range for healthy individuals (168-367µg/mL) as depicted in the assay protocol manual used for determination of serum VDBP levels and the range (300-600µg/mL) reported by (Blanton *et al.*, 2011) in their study.

In contrast , a randomized placebo-controlled trial reported lower 25(OH)D levels (P<0.001) and serum VDBP levels (P<0.001) in blacks compared to non-Blacks (Ponda *et al.*, 2014). Similarly, (Powe *et al.*, 2011) reported lower serum total 25(OH)D and VDBP levels in Blacks compared to White American subjects respectively. Though the results of the present study fall between those reported in Blacks and White Americans, they are more comparable with those of Whites than Black Americans.

Furthermore, the present study results showed a positive correlation between 25(OH)D and serum VDBP levels in females only but not in males. Similarly, lower 25(OH)D levels were strongly associated with DBP (rho= 0.57, p<0.001) in post-menarchal and adolescents European Americans and African Americans (Ashraf et al., 2014). However, correlation between 25(OH)D and serum VDBP levels in both male and females have been reported in other studies (Bolland *et al.*, 2007) whilst others found no correlation between 25(OH)D levels and serum VDBP in obese Caucasians (Holmlund-Suila et al., 2016). Since females had higher serum VDBP levels in the present study, then it could be predicted that their 25(OH)D levels would also be higher than males. However, most studies that have reported gender differences in 25(OH)D with males having higher levels than females (Bolland *et al.*, 2007); (Al-Dabhani et al., 2017).

It has been shown that obesity is one of the risk factors for vitamin D deficiency in diverse populations (Iqbal et al., 2017) and both obesity and vitamin D deficiency are also considered the risk factors for T2DM. The results of the present showed that females were significantly older and obese compared to males, with lower 25(OH)D levels being strongly correlated with BMI in females (P<0.0001). Similar to our observations a population-based study conducted in Norway reported a strong inverse association between low 25(OH)D levels and elevated BMI (Lagunova et al., 2011). A Meta-analysis conducted in Brazil reported a high prevalence of vitamin D deficiency with a significant association between low 25(OH)D levels and obesity irrespective of age and latitude (Pereira-Santos et al., 2015). Also, Caucasian women aged 18-31 years old had higher levels of fasting serum PTH and their 25(OH)D levels were associated with increased fat mass (Gunther et al., 2006). PTH is synthesized in response to low calcium levels and its concentrations are also increased in vitamin D deficiency states. Results of the present study have shown significant and inverse

correlation between 25(OH)D levels and increased PTH concentration in the female group, but this was not observed in the male group. In line with the present results, a population study has reported an association between obesity, low serum 25(OH)D levels, increased PTH and low active vitamin D (1.25-$(OH)_2D_3$) levels (Parikh et al., 2004). Increased PTH may be attributed by high frequency of vitamin D deficiency and degree of obesity in females compared to males respectively.

The causal effect of the relationship between vitamin D deficiency and obesity is poorly understood, although accumulating evidence suggests that vitamin D may be sequestrated into the increased adipose tissue compartments in the obese individuals thus limiting its bioavailability in the circulation (Bolland *et al.*, 2007). Low vitamin D status has been associated with glucose intolerance and occurrence of T2DM in several populations. Several studies have reported that patients with T2DM or glucose intolerance exhibit lower 25(OH)D levels as compared to healthy controls (Hurskainen et al., 2012); (Kostoglou-athanassiou *et al.*, 2013); (Lakshmi et al., 2015); (Rahman *et al.*, 2017). In contrast, 25(OH)D levels were significantly very low in both males and females in the hyper-glycaemic and normo-glycaemic groups in the present study.

The 25(OH)D levels in the present study were insufficient and serum VDBP levels did not differ among the glycaemic groups. These results suggest that this population may require vitamin D supplementation to maintain adequate 25(OH)D levels. Moreover, findings of the present are in accordance with those reported in a Saudi Arabian study which also observed low 25(OH)D levels in both T2DM patients and in healthy controls (Alhumaidi et al., 2013). The possible role of vitamin D deficiency in the pathogenesis of T2DM is not completely understood and evidence is sparse and inconclusive. The proposed mechanisms in which vitamin D deficiency predisposes to T2DM may be either through direct action on vitamin D receptor activation or indirectly via calcium hormones and inflammation (Thorand *et al.*, 2011); (Sung *et al.*, 2012).

Both T2DM and obesity are conditions that are associated with inflammation , with systemic inflammation being found to increase insulin resistance (Hribal et al., 2014). C-reactive protein (CRP) is an acute phase protein synthesized in response to inflammation or infection. A rise in CRP levels in the range of 3-10 mg/l indicates the presence of chronic low-grade inflammation and is frequently observed among obese subjects and is involved in the development of insulin resistance (Hribal *et al.*, 2014). Vitamin D has anti-inflammatory effects and low levels have been shown to be associated with a rise in CRP and increased proinflammatory cytokines such as IL-6

and TNFα, which impairs insulin signaling thus leading to systemic inflammation. The present study has demonstrated a strong inverse correlation between CRP and low 25(OH)D levels in females compared to males (P=0.0037), and also a strong inverse correlation with HOMA-IR in females only. Multiple linear regression revealed that CRP is a predictor of low 25(OH)D. This is evident because all females in the present study were obese, but males were overweight. Hence the increased CRP and its correlation with low 25(OH)D levels in females may be indicative of systemic inflammation leading to insulin resistance. Epidemiological studies have showed significant and inverse correlation between low 25(OH)D levels with raised fasting blood glucose and severity of insulin resistance (Zittermann, 2006); (Pittas *et al.*, 2010).

In the present study, low 25(OH)D levels were significantly and inversely correlated with high 2 hr BG, HbA1c, FBI, 2 hr BI and HOMA-IR in Normo-glycaemic females (All, P≤0.049). Low 25(OH)D levels were further significantly and inversely correlated with FBG and HbA1c (All, P≤0.011) and positively correlated with post 2 hr BI, (P=0.022) in screen-detected DM female subjects. In Nomo-glycaemic males low 25(OH)D levels were significantly and inversely correlated with post 2 hr BG (P=0.012) and inversely with FBI in screen-detected DM group (P=0.010). These results are consistent with those reported in a Bangladeshi population in which they found a significant negative correlation between 25(OH)D levels and fasting blood glucose among T2DM patients (Rahman *et al.*, 2017). Additionally, low 25(OH)D levels were negatively correlated with fasting plasma glucose and HOMA-IR in Egyptian patients (Clemente-Postigo *et al.*, 2015).

However, in the present study, multiple linear regression analysis revealed that FBG and FBI were not associated with low 25(OH)D levels in both normo-glycaemic and hyper-glycaemic males and females. These findings are inconsistent with data from the NHANES (III) study which reported an inverse association between serum 25(OH)D with fasting blood glucose, post 2 hr BG, fasting blood insulin and prevalence of T2DM (Scragg *et al.*, 2004). Another study also showed that low 25(OH)D levels were associated with post 2 hr blood glucose (Hurskainen *et al.*, 2012).

No correlation was observed between serum VDBP levels and glycaemic indicators in the present study. A significant positive correlation with FBI and post 2 hr BI was observed in screen-detected DM females, whereas in normo-glycaemic female

subjects there was a positive correlation between serum VDBP levels with post 2 hr BG and 2 hr BI (All, P≤0.084). Also, near-significant positive correlation with HbA1c was observed in normo-glycaemic males (P=0.097) and significant inverse correlation with post 2 hr BG was observed in pre-DM males (P=0.011).

In contrast to our results a more recent study in a Thai population have shown low serum VDBP levels in metabolic syndrome patients compared to healthy controls, and has demonstrated significant negative correlation of serum VDBP levels with systolic blood pressure, fasting blood glucose and age (Karuwanarint et al., 2018). Moreover, evidence from several studies suggest that vitamin D deficiency is associated with CVDs (Holick & Chen, 2008b) (Engelman *et al.*, 2010) through its effect on lipid profiles (Saedisomeolia et al., 2014). There is high prevalence of dyslipidaemia in patients with T2DM, which put them at a high risk for development of cardiovascular diseases (Vinodmahato et al., 2011).

Interestingly, the lipid profile parameters including triglycerides, LDL-C, and Cholesterol were significantly increased in both genders (All, males P≤0.0300, females P<0.0001) in the hyper-glycaemic sub-groups (All, p<0.0001), whereas HDL-C was significantly decreased in both males and females in the hyper-glycaemic sub-groups (All, p≤0.0308). Similarly, (Saedisomeolia *et al.*, 2014) has reported elevated TG, LDL-C, TC and reduced HDL-C levels in 108 Iranian patients with T2DM. In the present study, low serum 25(OH)D levels were significantly inversely correlated with increased TG, LDL-C, TC and reduced HDL-C in females, but in males there was a near significant inverse correlation with TG, LDL-C and TC only. Evidence suggest that hyperlipidaemia in females may be attributed to the effects of sex hormones on body fat distribution thus leading to differential altered lipoproteins compared to males (Sibley et al., 2006).

In the present study, low 25(OH)D levels were significantly and inversely correlated with TG, LDL-C and positively correlated with HDL-C in normo-glycaemic, prediabetic and known-diabetic females (All, P≤0.0490). However, in pre-diabetic male patients 25(OH)D levels were significantly and inversely correlated with HDL-C, but near significant correlation was observed in screen-detected DM males. Similarly, a study in Americans have shown that low 25(OH)D levels are inversely correlated with TC, TG, LDL-C and positively correlated with HDL-C (Gaddipati et al., 2011). In the present study multiple linear regression found a negative association between low 25(OH)D levels and increased LDL-C levels in both normo-glycaemic and hyper-glycaemic

groups. Unlike the findings of the present study, low 25(OH)D levels were positively associated with increased TC, HDL-C and LDL-C and negatively associated with TG among 1808 Norwegian subjects (Jorde et al., 2010). Vitamin D deficiency was significantly associated with increased diastolic blood pressure, TG, and reduced HDL-C in Caribbean population (Vélayoudom-céphise *et al.*, 2011), whereas in Iranians only increased TG was associated with low 25(OH)D levels (Saedisomeolia *et al.*, 2014).

In the present study serum VDBP (µg/mL) showed significant positive correlations with triglycerides (mmol/L) in normo-glycaemic and in pre-DM female subjects (All, P≤0.013). Furthermore, no correlation was observed with lipids in any of the females glycaemic sub-groups, but significant positive correlations with LDL-C (mmol/L) and Cholesterol (mmol/L) was observed in normo-glycaemic males (All, P≤0.002) and a significant positive correlation with HDL-C (P=0.018) and a near-significant positive correlation with Cholesterol (mmol/L) (P=0.103) in screen-detected DM males. Multiple linear regression analysis revealed an association between low 25(OH)D and serum VDBP levels in hyperglycaemic females only. There is a lack of data in the literature on the association of serum VDBP and diabetes with data being focused more on the association between VDBP polymorphisms and increased risk for T2DM.

As stated earlier, evidence from the literature has shown that various risk factors are implicated in the onset and progression of T2DM. Moreover, several genes involved in its metabolic pathway have been regarded as good candidates for its onset (Barroso, 2005), among these, VDR gene is considered as an important candidate gene for susceptibility to T2DM. This is due to expression of VDR on various cells involved in the regulation of glucose metabolism (Palomer *et al.*, 2008), and the fact that vitamin D modulates expression of insulin receptor genes, insulin secretion and exerts its action on target cells through binding to VDR (Al-Daghri, Al-Attas, Alokail, Alkharfy, Draz, *et al.*, 2012). Hence variation in VDR may possibly contribute to susceptibility to T2DM. The present study has investigated the association between VDR polymorphisms Fok1 (rs2228570), Taq1 (rs731236) and Apa1 (rs7975232) in diabetic (T2DM) and non-diabetic patients in the mixed ancestry population group.

The results of the present study have shown non-significant differences in the allelic and genotypic frequencies of the VDR gene (Fok1 (A>G), Apa1 (C>A), and Taq1

(A>G)) SNPs between T2DM and non-diabetic patients (Table 5.10A and B). Interestingly, these alleles and genotypes frequencies were similar to those reported in this population previously (Babb, van der Merwe, Beyers, Pheiffer, Walzl, Duncan, van Helden & Hoal, 2007). Fok1 variant allele G encodes 424 amino acid base long protein compared to variant allele A which encodes 427 amino acid long protein (Uitterlinden *et al.*, 2004); (Palomer *et al.*, 2008). Studies have suggested that shorter proteins have higher transcriptional activity which in turn increases binding affinity for active vitamin D (1.25-$(OH)_2$D) (Palomer *et al.*, 2008). Hence this can reduce the risk for T2DM by improving insulin sensitivity, insulin secretion and insulin resistance. Results of the present study have further shown that variant allele G of the Fok1 SNPs was not associated with T2DM (75.4%), compared to non-diabetics (73.9%), and the variant genotype GG frequency of the Fok1 SNPs in T2DM (58.5%) was not significantly different from Non-diabetics (55.1%).

In contrast to our results , a more recent study has shown an association of the variant G allele and variant AG and GG genotypes of the Fok1 SNPs and variant allele T and TT genotypes of the Bsm1 SNP with increased risk for T2DM in an Emirati population (Al *et al.*, 2018). These results were corroborated in a meta-analysis of cross-sectional studies in Caucasians and East Asians which showed an association between Fok1 SNPs with increased risk for T2DM more especially in East Asians (Wang *et al.*, 2012). Also, Fok1 SNPs were associated with an increased risk for T2DM in Kashmir Valley population (Malik *et al.*, 2017) from India. However, other studies failed to reproduce the association between VDR SNPs and T2DM. For example, (Banerjee *et al.*, 2009) using Fok1, Apa1 and Bsm1 demonstrated that there is no association between polymorphisms in the VDR gene with T2DM risk in North Indians. Moreover, (Cyganek *et al.*, 2006) also failed to demonstrate such an association in a Polish population.

Previous studies have shown that polymorphisms within intron 8 and exon 9 of the VDR affect the expression of this protein (Ogunkolade *et al.*, 2002). For example, A allele of the Taq1 SNP was associated with an increased VDR protein expression in Saudi Arabians (Al-Daghri *et al.*, 2012). In the present study, Apa1 variant allele A was not associated with T2DM (61.3%) compared to non-diabetics (61.5%), and there was non-significant difference in the variant genotype AA frequency between T2DM (40.1%) and non-diabetics (38.0%). In contrast, Apa1 genotypes were associated with T2DM in an American population (Oh et al., 2002). Moreover, In the present study,

Taq1 variant allele G was not associated with T2DM (28.5%) compared to Non-diabetics (28.2%), there were non-significant difference in the variant genotype GG frequency between T2DM (6.9%) and Non-diabetics (8.5%).

On the contrary, the variant AG genotype of the Taq1 SNP and variant genotype CC and CT of the Bsm1 SNP were associated with increased risk for T2DM in Saudi Arabian patients compared to healthy controls (Al-Daghri *et al.*, 2012). Similarly, variant A allele and AG allele of the Taq1 polymorphism were associated with increased risk for T2DM in obese patients in Iraq (Al-darraji *et al.*, 2017). Even though results of the present study are in accordance with those shown in North Indians (Banerjee *et al.*, 2009), Polish (Cyganek *et al.*, 2006) and French (Dilmec *et al.*, 2010) which demonstrated no association, but they have presented some differences from the results of the present study. For example, in Caucasians the variant genotype AA of the Apa1 polymorphism was marginally high in T2DM patients compared to controls (Oh et al., 2002).

Hence, it could be summarised that Bsm1 and Taq1 polymorphisms are important in the pathogenesis of T2DM through their effects on VDR gene expression level. The discrepancies of the results reported in the literature and those of the present study could possibly be explained by genetic differences and environmental exposure. Since majority of studies were performed in homogenous populations groups, the present study in contrast was performed in a population that comprised of many ancestry descendants including Khoisan (32-43%), Bantu speaking African (20-36%), European (21-28%) and Asian (9-11%) (de Wit et al., 2010).

VDR gene variants are associated with metabolic complications involved in the development of T2DM such as dyslipidaemia, obesity and other cardiovascular risk factors. For example, variant AA genotypes of the Apa1 polymorphisms were associated with glucose intolerance in T2D patients (Oh et al., 2002). Taq1 and Bsm1 genotypes were frequent in T2DM patients compared to controls and were associated with increased total Cholesterol and lower HDL-C in Saudi Arabians (Al-Daghri *et al.*, 2012). In addition, variant genotype FF of the Fok1 SNPs was significantly associated with increased TC, TG, LDL-C and HDL-C in Moroccans patients with T2DM (Abdeltif *et al.*, 2014). VDR polymorphisms were not associated with either vitamin D deficiency/insufficiency in the present study.

However, vitamin D prevalence of vitamin D deficiency was associated with the variant f (T) allele of the Fok1 SNP and aa (AA) genotype of the Apa1 SNP in Caribbean patients (Vélayoudom *et al.*, 2011). These results suggest that the f allele of the Fok1 SNP and AA genotype of the Apa1 may be protective against vitamin D deficiency. The present study demonstrated no significance difference in the genotype or allele frequency distribution of Fok1 (rs2228570), Apa1 (rs7975232) or Taq1 (rs731236) SNPs between subjects with optimal Vitamin D (25(OH)D ng/mL) levels and those with insufficient/deficient levels ($P \geq 0.2036$ and $P \geq 0.6347$ respectively). These trends were also observed when serum VDBP levels were evaluated against Fok1, Apa1 and Taq1 genotypes. Also, there was no significance difference in the SNPs frequencies in obese and overweight participants. SNP frequencies were insignificantly different among patients with moderate/severe insulin resistance compared to normal insulin resistance participants in the present study. These results suggest that VDR seems not to be a suitable candidate for susceptibility to T2DM mellitus in the mixed ancestry population group.

The present study had limitations. The study design was cross-sectional in nature hence association found does not demonstrate a causal relationship. A detailed food frequency questionnaire for evaluation of food intake containing vitamin D was not administered. Seasonal variation of serum vitamin D levels was not taken into consideration. The study had a high female participation compared to males. The present study was strengthened by a relatively large sample size. It has comprised of varying glycaemic groups. For example, the hyper-glycaemic sub-groups comprised of pre-diabetics, screen-detected DM and known DM. The present study was also strengthened by the fact that we analyzed both vitamin D levels, its transporter in the circulation (vitamin D binding proteins) and the polymorphisms of its receptors on target tissue/cells.

Chapter 7 Conclusion

The findings of the present study have significant clinical and public health implications. The population studied is known to have a high prevalence of obesity, hypertension and T2DM. Compelling evidence from epidemiological and observational studies have shown that vitamin D deficiency is common in obese individuals and is associated with increased risk for insulin resistance and T2DM. In the present studied population vitamin D deficiency and insufficiency were highly prevalent. In addition, Obesity was highly prevalent in females compared to males. Overall vitamin D levels were insufficient, vitamin D levels were low in both normo-glycaemic and hyper-glycaemic males and females while serum VDBP was within the normal range.

Serum VDBP was associated with low 25(OH)D in hyper-glycaemic females only. Alleles and genotype frequencies of the VDR gene SNPs (Fok1, Apa1 and Taq1) were not significantly different among the normo-glycaemic and hyper-glycaemic groups. Therefore, this finding shows that VDR gene SNPs are not associated with T2DM in the studied population. Low 25(OH)D was associated with increased LDL-C and PTH in both male and females irrespective of T2DM. low 25(OH)D was not associated with any of the T2DM phenotypes and metabolic traits. Overall conclusion, Low 25(OH)D levels were not associated with serum vitamin D binding protein and VDR polymorphisms in T2DM patients in the mixed ancestral population group.

Vitamin D supplementation may carefully be recommended to increase the 25(OH)D levels and to observe its effect on the regulation of glycaemic status in the studied population. In addition, further genetic studies should be designed to identify genes associated with increased susceptibility for T2DM and obesity in this population. Other studies should be conducted to replicate these findings of the present study.

References

Abdeltif, E., Benrahma, H., Charoute, H., Barakat, H. & Rouba, H. 2014. Vitamin D receptor gene polymorphisms and vitamin D status and susceptibility to type 2 diabetes mellitus in Moroccans patients. *Internation Journal of Scientific and research Publication*. 4(8):1–9.

Al-Dabhani, K., Tsilidis, K.K., Murphy, N., Ward, H.A., Elliott, P., Riboli, E., Gunter, M. & Tzoulaki, I. 2017. Prevalence of vitamin D deficiency and association with metabolic syndrome in a Qatari population. *Nutrition & Diabetes*. 7(4):e263.

Al-daghri, N.M., Al-attas, O.S., Alkharfy, K.M., Khan, N., Khader, A., Vinodson, B., Ghouse, M., Ansari, A., et al. 2014. Association of VDR-gene variants with factors related to the metabolic syndrome , type 2 diabetes and vitamin D de fi ciency. *Gene*. 542(2):129–133.

Al-Daghri, N.M., Al-Attas, O., Alokail, M.S., Alkharfy, K.M., Draz, H.M., Agliardi, C., Mohammed, A.K., Guerini, F.R., et al. 2012. Vitamin D Receptor Gene Polymorphisms and HLA DRB1*04 Cosegregation in Saudi Type 2 Diabetes Patients. *The Journal of Immunology*. 188(3):1325–1332.

Al-Daghri, N.M., Al-Attas, O.S., Alokail, M.S., Alkharfy, K.M., El-Kholie, E., Yousef, M., Al-Othman, A., Al-Saleh, Y., et al. 2012. Increased vitamin D supplementation recommended during summer season in the gulf region: A counterintuitive seasonal effect in vitamin D levels in adult, overweight and obese Middle Eastern residents. *Clinical Endocrinology*. 76(3):346–350.

Al-darraji, S.Z., Al-azzawie, H.F. & Al-kharsani, A.M. 2017. Journal of Diabetes & Metabolism Associations between TaqI Polymorphisms in Vitamin D Receptor Gene and Type 2 Diabetes Mellitus in Obese Iraqi Population. *Diabetes and Metabolism*. 8(4):8–11.

Al, H., El, S., Chehadeh, H., Abdel-wareth, L., Haq, A., Jelinek, H.F., Elghazali, G. & Al, F. 2018. Journal of Steroid Biochemistry & Molecular Biology Vitamin D receptor gene polymorphisms among Emirati patients with type 2 diabetes mellitus. *Journal of Steroid Biochemistry and Molecular Biology*. 175(2018):119–124.

Alhumaidi, M., Agha, A. & Dewish, M. 2013. Vitamin d deficiency in patients with type-2 diabetes mellitus in southern region of saudi arabia. *Maedica*. 8(3):231–6. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/24371490 [2018, October 04].

Almgren, P., Lehtovirta, M., Isomaa, B., Sarelin, L., Taskinen, M.R., Lyssenko, V., Tuomi, T., Groop, L., et al. 2011. Heritability and familiality of type 2 diabetes and related quantitative traits in the Botnia Study. *Diabetologia*. 54(11):2811–2819.

Amercan Diabetes Association. 2010. DEFINITION AND DESCRIPTION OF DIABETES OTHER CATEGORIES OF GLUCOSE. *Diabetes Care*. 33(supplement 1):S62-68.

American Diabetes Association. 2014. Diagnosis and classification of diabetes mellitus. *Diabetes Care*. 37(SUPPL.1):81–90.

Appel, S.J., Wadas, T.M., Rosenthal, R.S. & Ovalle, F. 2009. Latent autoimmune diabetes of adulthood (LADA): An often misdiagnosed type of diabetes mellitus. *Journal of the American Academy of Nurse Practitioners*. 21(3):156–159.

Ashraf, A.P., Huisingh, C., Alvarez, J.A., Wang, X. & Gower, B.A. 2014. Insulin Resistance Indices Are Inversely Associated With Vitamin D Binding Protein Concentrations. *Journal of Clinical Endocrinol Metablism*. 99(1):178–183.

Babb, C., van der Merwe, L., Beyers, N., Pheiffer, C., Walzl, G., Duncan, K., van Helden, P. & Hoal, E.G. 2007. Vitamin D receptor gene polymorphisms and sputum conversion time in pulmonary tuberculosis patients. *Tuberculosis*. 87(4):295–302.

Bai, X.-Y., Miao, D., Goltzman, D. & Karaplis, A.C. 2003. The Autosomal Dominant Hypophosphatemic Rickets R176Q Mutation in Fibroblast Growth Factor 23 Resists Proteolytic Cleavage and Enhances *in Vivo* Biological Potency. *Journal of Biological Chemistry*. 278(11):9843–9849.

Banerjee, M., Bid, H., Konwar, R., Aggarwal, C., Gautam, S., Saxena, M. & Nayak, Vl. 2009. Vitamin D receptor (FokI, BsmI and TaqI) gene polymorphisms and type 2 diabetes mellitus : A North Indian study. *Indian Journal of Medical*

Sciences. 63(5):187.

Barbour, L.A., McCurdy, C.E., Hernandez, T.L., Kirwan, J.P., Catalano, P.M. & Friedman, J.E. 2007. Cellular mechanisms for insulin resistance in normal pregnancy and gestational diabetes. *Diabetes Care*. 30(SUPPL. 2):s112–s119.

Barroso, I. 2005a. Genetics of Type 2 diabetes. *Diabetic Medicine*. 22(5):517–535.

Barroso, I. 2005b. Genetics of Type 2 diabetes. *Diabetic Medicine*. 22(5):517–535.

Barroso, I., Luan, J., Sandhu, M.S., Franks, P.W., Crowley, V., Schafer, A.J., O'Rahilly, S. & Wareham, N.J. 2006. Meta-analysis of the Gly482Ser variant in PPARGC1A in type 2 diabetes and related phenotypes. *Diabetologia*. 49(3):501–505.

Bellia, A., Garcovich, C., D'Adamo, M., Lombardo, M., Tesauro, M., Donadel, G., Gentileschi, P., Lauro, D., et al. 2013. Serum 25-hydroxyvitamin D levels are inversely associated with systemic inflammation in severe obese subjects. Internal and Emergency Medicine. 8(1):33–40.

Bener, A., Yousafzai, M.T. & Al-hamaq, A.O. 2012. Familial aggregation of T2DM among Arab diabetic population. *International Journal of Diabetes in Developing Countries*. 32(2):90–92.

Benrahma, H., Arfa, I., Charif, M. & Bounaceur, S. 2011. Maternal Effect and Familial Aggregation in a Type 2 Diabetic Moroccan Population. *Journal Community Health*. 36(6):943–948.

Berraho, M., El Achhab, Y., Benslimane, A., El Rhazi, K.E., Chikri, M. & Nejjari, C. 2012. Hypertension and type 2 diabetes: A cross-sectional study in Morocco (EPIDIAM study). *Pan African Medical Journal*. 11:52.

Bikle, D.D. 2014. Review Vitamin D Metabolism , Mechanism of Action , and Clinical Applications. *Chemistry & Biology*. 21(3):319–329.

Blanton, D., Han, Z., Bierschenk, L., Linga-Reddy, M. V, Wang, H., Clare-Salzler, M., Haller, M., Schatz, D., et al. 2011. Reduced serum vitamin D-binding protein levels are associated with type 1 diabetes. *Diabetes*. 60(10):2566–2570.

Bodnar, L.M., Siega-riz, A.M., Simhan, H.N., Himes, K.P. & Abrams, B. 2010. Severe

obesity , gestational weight gain , and adverse birth outcomes 1 – 3. *Am J Clin Nutr*. 91:1642–1648.

Bolland, M.J., Grey, A.B., Ames, R.W., Horne, A.M., Mason, B.H., Wattie, D.J., Gamble, G.D., Bouillon, R., et al. 2007. Age-, gender-, and weight-related effects on levels of 25-hydroxyvitamin D are not mediated by vitamin D binding protein. *Clinical Endocrinology*. 67(2):259–264.

Bouillon, R., Eelen, G., Verlinden, L., Mathieu, C., Carmeliet, G. & Verstuyf, A. 2006. Vitamin D and cancer. *The Journal of Steroid Biochemistry and Molecular Biology*. 102(1–5):156–162.

Braithwaite, V.S., Jones, K.S., Schoenmakers, I., Silver, M., Prentice, A. & Hennig, B.J. 2015. Vitamin D binding protein genotype is associated with plasma 25OHD concentration in West African children. *Bone*. 74:166–70.

Braun, A., Bichlmaier, R. & Cleve, H. 1992. Molecular analysis of the gene for the human vitamin-D-binding protein (group-specific component): allelic differences of the common genetic GC types. *Human Genetics*. 89(4):401–406.

Cerf, M.E. 2013. Beta Cell Dysfunction and Insulin Resistance Beta cell dysfunction and insulin resistance. *Frontiers in endocrinology*. 4(June):1–12.

Chang, A.M. & Halter, J.B. 2003. Aging and insulin secretion. *American Journal of Physiology-Endocrinology and Metabolism*. 284(1):E7–E12.

Chen, T.C., Chimeh, F., Lu, Z., Mathieu, J., Person, K.S., Zhang, A., Kohn, N., Martinello, S., et al. 2007. Factors that influence the cutaneous synthesis and dietary sources of vitamin D. *Archives of Biochemistry and Biophysics*. 460(2):213–217.

Chiu, K.C., Chu, A., Liang, V., Go, W. & Saad, M.F. 2004. *Hypovitaminosis D is associated with insulin resistance and cell dysfunction 1-3*. [Online], Available: https://academic.oup.com/ajcn/article-abstract/79/5/820/4690192 [2018, October 23].

Choi, H.S., Kim, K.-A., Lim, C.-Y., Rhee, S.Y., Hwang, Y.-C., Kim, K.M., Kim, K.J., Rhee, Y., et al. 2011. Low Serum Vitamin D Is Associated with High Risk of Diabetes in Korean Adults. *Journal of Nutrition*. 141(8):1524–1528.

Christakos, S., Ajibade, D. V., Dhawan, P., Fechner, A.J. & Mady, L.J. 2010. Vitamin D: Metabolism. *Endocrinology and Metabolism Clinics of North America*. 39(2):243–253.

Christakos, S., Barletta, F., Huening, M., Dhawan, P., Liu, Y., Porta, A. & Peng, X. 2003. Vitamin D target proteins: Function and regulation. Journal of Cellular Biochemistry. 88(2):238–244.

Chu, S., Callaghan, W., Kim, S., Schmid, C., Lau, J., England, L. & Dietz, P. 2007. Maternal Obesity and Risk of Gestational DIabetes Mellitus. *Diabetes Care*. 30(8):2070–2076.

Chun, R.F., Peercy, B.E., Orwoll, E.S., Nielson, C.M., Adams, J.S. & Hewison, M. 2014. Vitamin D and DBP: The free hormone hypothesis revisited. *The Journal of Steroid Biochemistry and Molecular Biology*. 144:132–137.

Clemente-Postigo, M., Muñoz-Garach, A., Serrano, M., Garrido-Sánchez, L., Bernal-López, M.R., Fernández-García, D., Moreno-Santos, I., Garriga, N., et al. 2015. Serum 25-Hydroxyvitamin D and Adipose Tissue Vitamin D Receptor Gene Expression: Relationship With Obesity and Type 2 Diabetes. *The Journal of Clinical Endocrinology & Metabolism*. 100(4):E591–E595.

Cooke, D.W. & Plotnick, L. 2008. Management of Diabetic Ketoacidosis in Children and.

Corriere, M., Rooparinesingh, N. & Kalyani, R.R. 2013. Epidemiology of diabetes and diabetes complications in the elderly: an emerging public health burden. *Current diabetes reports*. 13(6):805–13.

Cowie, C.C., Rust, K.F., Ford, E.S., Eberhardt, M.S., Byrd-Holt, D.D., Li, C., Williams, D.E., Gregg, E.W., et al. 2009. Full accounting of diabetes and pre-diabetes in the U.S. population in 1988-1994 and 2005-2006. *Diabetes care*. 32(2):287–94.

Cyganek, K., Mirkiewicz-Sieradzka, B., Malecki, M.T., Wolkow, P., Skupien, J., Bobrek, J., Czogala, M., Klupa, T., et al. 2006. Clinical risk factors and the role of VDR gene polymorphisms in diabetic retinopathy in Polish type 2 diabetes patients. *Acta Diabetol*. 43(4):114–119.

Daniel, M.J. 2011. Lipid management in patients with type 2 diabetes. *American health & drug benefits*. 4(5):312–22. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/25126359 [2019, February 01].

Davison, G.M., Nkambule, B.B., Mkandla, Z., Hon, G.M., Kengne, A.P., Erasmus, R.T. & Matsha, T.E. 2017. Platelet, monocyte and neutrophil activation and glucose tolerance in South African Mixed Ancestry individuals. *Scientific Reports*. 7(January):1–9.

Deleskog, A., Hilding, A., Brismar, K., Hamsten, A., Efendic, S. & Östenson, C.G. 2012. Low serum 25-hydroxyvitamin D level predicts progression to type 2 diabetes in individuals with prediabetes but not with normal glucose tolerance. *Diabetologia*. 55(6):1668–1678.

DeLuca, H.F. 2004. Overview of general physiologic features and functions of vitamin D. *The American journal of clinical nutrition*. 80(6 Suppl):1689–1696.

De Wit, E., Delport, W., Rugamika, C.E., Meintjes, A., Möller, M., van Helden, P.D., Seoighe, C. & Hoal, E.G. 2010. Genome-wide analysis of the structure of the South African Coloured Population in the Western Cape. Human Genetics. 128(2):145–153.

Dilmec, F., Uzer, E., Akkafa, F., Kose, E. & van Kuilenburg, A.B.P. 2010. Detection of VDR gene ApaI and TaqI polymorphisms in patients with type 2 diabetes mellitus using PCR-RFLP method in a Turkish population. *Journal of Diabetes and its Complications*. 24(3):186–191.

El-Atat, F., McFarlane, S.I. & Sowers, J.R. 2004. Diabetes, hypertension, and cardiovascular derangements: Pathophysiology and management. *Current Hypertension Reports*. 6(3):215–223.

Engelman, C.D., Meyers, K.J., Ziegler, J.T., Taylor, K.D., Palmer, N.D., Haffner, S.M., Fingerlin, T.E., Wagenknecht, L.E., et al. 2010. Genome-wide association study of vitamin D concentrations in Hispanic Americans: The IRAS Family Study. *Journal of Steroid Biochemistry and Molecular Biology*. 122(4):186–192.

Erasmus, R.T., Blanco, E.B., Okesina, A.B., Arana, J.M., Gqweta, Z. & Matsha, T. 2001. Importance of family history in type 2 black South African diabetic

patients. *Postgraduate Medical Journal*. 77(907):323–325.

Erasmus, R.T., Soita, D.J., Hassan, M.S., Blanco-Blanco, E., Vergotine, Z., Kengne, A.P. & Matsha, T.E. 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African coloured population: Baseline data of a study in Bellville, Cape Town. *South African Medical Journal*. 102(11):841–844.

Erber, E., Hopping, B.N., Grandinetti, A., Park, S., Kolonel, L.N. & Maskarinec, G. 2009. Dietary Patterns and Risk for Diabetes : The Multiethnic Cohort. *Dietary Patterns and Diabetes*. 10(August):1–14.

van Etten, E., Verlinden, L., Giulietti, A., Ramos-Lopez, E., Branisteanu, D.D., Ferreira, G.B., Overbergh, L., Verstuyf, A., et al. 2007. The vitamin D receptor gene FokI polymorphism: Functional impact on the immune system. *European Journal of Immunology*. 37(2):395–405.

Van Etten, E. & Mathieu, C. 2005. Immunoregulation by 1,25-dihydroxyvitamin D3: Basic concepts. *Journal of Steroid Biochemistry and Molecular Biology*. 97(1–2):93–101.

Feldman, R.D., Fitchett, D., Hegele, R.A. & Poulter, N.R. 2018. Type 2 Diabetes and the Reduction of Cardiovascular Risk: Sorting Out the Actors and the Roles. *Canadian Journal of Cardiology*. 34(5):532–535.

Felsenfeld, A.J., Rodríguez, M. & Aguilera-Tejero, E. 2007. Dynamics of parathyroid hormone secretion in health and secondary hyperparathyroidism. *Clinical journal of the American Society of Nephrology : CJASN*. 2(6):1283–305.

Florez, J.C., Hirschhorn, J. & Altshuler, D. 2003. T HE I NHERITED B ASIS OF D IABETES M ELLITUS : Implications for the Genetic Analysis of Complex Traits. *Annual Review of Genomics and Human Genetics*. 4(1):257–291.

Forouhi, N.G., Luan, J., Cooper, A., Boucher, B.J. & Wareham, N.J. 2008. The Medical Research Council Ely Prospective Study 1990 –2000. *Diabetes*. 57(10):2619–2625.

Freeman, J., Wilson, K., Spears, R., Shalhoub, V. & Sibley, P. 2014. Influence of Vitamin D Binding Protein on Accuracy of 25-Hydroxyvitamin D Measurement Using the ADVIA Centaur Vitamin D Total Assay. *International journal of*

endocrinology. 2014(ID691679):1–13.

Freemantle, N., Holmes, J., Hockey, A. & Kumar, S. 2008. How strong is the association between abdominal obesity and the incidence of type 2 diabetes ? *International Journal of Clinical Practice*. 62(9):1391–1396.

Fruchart, J.-C., Sacks, F.M., Hermans, M.P., Assmann, G., Brown, W.V., Ceska, R., Chapman, M.J., Dodson, P.M., et al. 2008. The Residual Risk Reduction Initiative: a call to action to reduce residual vascular risk in dyslipidaemic patients. *Diabetes and Vascular Disease Research*. 5(4):319–335.

Gaddipati, V.C., Bailey, B.A., Kuriacose, R., Copeland, R.J., Manning, T. & Peiris, A.N. 2011. The relationship of vitamin D status to cardiovascular risk factors and amputation risk in veterans with peripheral arterial disease. *J Am Med Dir Assoc*. 12:58–61.

Gajardo, A., Srur, A., Wallbaum, M. & Erazo, M. 2017. The population impact of obesity , sedentary lifestyle , and tobacco and alcohol consumption on the prevalence of type 2 diabetes : Analysis of a health population survey in Chile , 2010. 1–12.

Ghaderpanahi, M., Fakhrzadeh, H., Sharifi, F., Badamchizade, Z., Mirarefin, M., Ebrahim, R.P., Ghotbi, S., Nouri, M., et al. 2011. Association of physical activity with risk of type 2 diabetes. *Iranian journal of public health*. 40(1):86–93. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/23113060 [2018, September 27].

Glans, F., Elgzyri, T., Shaat, N., Lindholm, E., Apelqvist, J. & Groop, L. 2008. Immigrants from the Middle-East have a different form of Type 2 diabetes compared with Swedish patients. *Diabetic Medicine*. 25(3):303–307.

Gregory, J.M., Moore, D.J. & Simmons, J.H. 2013. Type 1 Diabetes Mellitus. *Pediatrics in Review*. 34(5):203–215.

Grimnes, G., Emaus, N., Joakimsen, R.M., Figenschau, Y., Jenssen, T., Njolstad, I., Schirmer, H. & Jorde, R. 2010. Baseline serum 25-hydroxyvitamin D concentrations in the Tromso Study 1994-95 and risk of developing type 2 diabetes mellitus during 11 years of follow-up. *Diabet Med*. 27(10):1107–1115.

Gunther, C.W., Legowski, P.A., Lyle, R.M., Weaver, C.M., McCabe, L.D., McCabe, G.P., Peacock, M. & Teegarden, D. 2006. Parathyroid hormone is associated with decreased fat mass in young healthy women. *Int J Obes (Lond).* 30(1):94–99.

Haemer, M.A., Grow, H.M., Fernandez, C., Lukasiewicz, G.J., Rhodes, E.T., Shaffer, L.A., Sweeney, B., Woolford, S.J., et al. 2014. Addressing Prediabetes in Childhood Obesity Treatment Programs: Support from Research and Current Practice. *Childhood obesity*. 10(4):292–303.

Hagenau, T., Vest, R., Gissel, T.N., Poulsen, C.S., Erlandsen, M., Mosekilde, L. & Vestergaard, P. 2009. Global vitamin D levels in relation to age, gender, skin pigmentation and latitude: An ecologic meta-regression analysis. *Osteoporosis International*. 20(1):133–140.

Hara, K., Horikoshi, M., Kitazato, H., Lto, C., Noda, M., Ohashi, J., Froguel, P., Tokunaga, K., et al. 2006. Hepatocyte nuclear factor-4 alpha P2 promoter haplotypes are associated with type 2 diabetes in the Japanese population. *Diabetes*. 55(5):1260–1264.

Hari Kumar, K.V.S. & Modi, K.D. 2014. Twins and endocrinology. *Indian journal of endocrinology and metabolism*. 18(Suppl 1):S48-52.

Haussler, M.R., Haussler, C.A., Bartik, L., Whitfield, G.K., Hsieh, J.C., Slater, S. & Jurutka, P.W. 2008. Vitamin D receptor: Molecular signaling and actions of nutritional ligands in disease prevention. *Nutrition Reviews*. 66(SUPPL.2):S98–S112.

Haussler, M.R., Jurutka, P.W., Mizwicki, M. & Norman, A.W. 2011. Vitamin D receptor (VDR)-mediated actions of 1α,25(OH)$_2$vitamin D$_3$: genomic and non-genomic mechanisms. *Best practice & research. Clinical endocrinology & metabolism*. 25(4):543–59.

Heijboer, A.C., Blankenstein, M.A., Kema, I.P. & Buijs, M.M. 2012. Accuracy of 6 routine 25-hydroxyvitamin D assays: Influence of vitamin D binding protein concentration. *Clinical Chemistry*. 58(3):543–548.

Hitman, G.A., Mannan, N., McDermott, M.F., Aganna, E., Ogunkolade, B.W., Hales,

C.N., Boucher, B.J., Mannan, N., et al. 1998. Vitamin D receptor gene polymorphisms influence insulin secretion in Bangladeshi Asians. *Diabetes*. 47(4):688–90.

Holick, M.F. 1994. McCollum award lecture, 1994: Vitamin D - New horizons for the 21st century. *American Journal of Clinical Nutrition*. 60(4):619–630.

Holick, M.F. 2007. Vitamin D Deficiency. *New England Journal of Medicine*. 357(3):266–281.

Holick, M.F. 2009. Vitamin D Status: Measurement, Interpretation, and Clinical Application. *Annals of Epidemiology*. 19(2):73–78.

Holick, M.F. 2011. Vitamin D. *Journal of Investigative Medicine*. 59(6):872–880.

Holick, M.F. & Chen, T.C. 2008a. Vitamin D deficiency: a worldwide problem with health consequences–. *The American Journal of Clinical Nutrition*. 87(4):1080S–1086S.

Holick, M.F. & Chen, T.C. 2008b. Vitamin D deficiency: a worldwide problem with health consequences. *The American Journal of Clinical Nutrition*. 87(4):1080S–1086S.

Holick, M.F., Binkley, N.C., Bischoff-ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H. & Weaver, C.M. 2011. Evaluation , Treatment , and Prevention of Vitamin D Deficiency : an Endocrine Society Clinical Practice Guideline. *J clin endocrinol metab, july 2011*. 96(7):1911–1930.

Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H. & Weaver, C.M. 2011. Evaluation, treatment, and prevention of vitamin D deficiency: An endocrine society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism*. 96(7):1911–1930.

Holmlund-Suila, E., Pekkinen, M., Ivaska, K.K., Andersson, S., Mäkitie, O. & Viljakainen, H. 2016. Obese young adults exhibit lower total and lower free serum 25-hydroxycholecalciferol in a randomized vitamin D intervention. *Clinical Endocrinology*. 85(3):378–385.

Holt, R.I.G. 2004. Diagnosis , epidemiology and pathogenesis of diabetes mellitus : an update for psychiatrists. *British journal of psychiatry*. 4(Suppl.47):55–63.

Hribal, M.L., Fiorentino, T.V. & Sesti, G. 2014. Role of C reactive protein (CRP) in leptin resistance. *Current pharmaceutical design*. 20(4):609–15.

Hu, F.B., Liu, S. & van Dam, R.M. 2001. Diet and risk of Type II diabetes: the role of types of fat and carbohydrate. *Diabetologia*. 44(7):805–817.

Hurskainen, A.-R., Virtanen, J.K., Tuomainen, T.-P., Nurmi, T. & Voutilainen, S. 2012. Association of serum 25-hydroxyvitamin D with type 2 diabetes and markers of insulin resistance in a general older population in Finland. *Diabetes/Metabolism Research and Reviews*. 28(5):418–423.

Iqbal, A.M., Dahl, A.R., Lteif, A. & Kumar, S. 2017. Vitamin D Deficiency: A Potential Modifiable Risk Factor for Cardiovascular Disease in Children with Severe Obesity. Children. 4(9):1–10.

Jorde, R., Figenschau, Y., Hutchinson, M., Emaus, N. & Grimnes, G. 2010. High serum 25-hydroxyvitamin D concentrations are associated with a favorable serum lipid profile. *European Journal of Clinical Nutrition*. 64(12):1457–1464.

Joseph, J.J., Echouffo-tcheugui, J.B., Golden, S.H., Chen, H., Jenny, N.S., Carnethon, M.R., Jr, D.J., Burke, G.L., et al. 2016. Physical activity , sedentary behaviors and the incidence of type 2 diabetes mellitus : the Multi-Ethnic Study of Atherosclerosis (MESA). *BMJ open diabetes research & care*. 4(e0000185):1–12.

Kahn, S.E., Hull, R.L. & Utzschneider, K.M. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature*. 444(December):840–846.

Karpe, F., Dickmann, J.R. & Frayn, K.N. 2011. Fatty Acids, Obesity, and Insulin Resistance: Time for a Reevaluation. *Diabetes*. 60(October):2441–2449.

Karuwanarint, P., Phonrat, B., Tungtrongchitr, A., Suriyaprom, K., Chuengsamarn, S., Schweigert, F.J. & Tungtrongchitr, R. 2018. Vitamin D-binding protein and its polymorphisms as a predictor for metabolic syndrome. *Biomarkers in Medicine*. 12(5):465–473.

Kostoglou-athanassiou, I., Athanassiou, P. & Gkountouvas, A. 2013. Vitamin D and glycemic control in diabetes mellitus type 2. *Therapeutic Advances in Endocrinology and Metabolism*. 4(4):122–128.

Kull, M., Kallikorm, R., Tamm, A. & Lember, M. 2009. Seasonal variance of 25-(OH) vitamin D in the general population of Estonia, a Northern European country. *BMC Public Health*. 9(22):1–8.

Kumar, R. & Thompson, J.R. 2011. The regulation of parathyroid hormone secretion and synthesis. *Journal of the American Society of Nephrology : JASN*. 22(2):216–24.

Lagunova, Z., Porojnicu, A.C., Lindberg, F.A., Aksnes, L. & Moan, J. 2011. Vitamin D status in Norwegian children and adolescents with excess body weight. *Pediatric Diabetes*. 12(2):120–126.

Lakshmi, G. & Silambanan, S. 2015. Vitamin D Status in Type 2 Diabetes Mellitus. *iInternational Journal of Clinical Biochemistry and Research*. 2(3):140–142.

Lampasona, V., Petrone, A., Tiberti, C., Capizzi, M., Spoletini, M., Di Pietro, S., Songini, M., Bonicchio, S., et al. 2010. Zinc transporter 8 antibodies complement GAD and IA-2 antibodies in the identification and characterization of adult-onset autoimmune diabetes: Non Insulin Requiring Autoimmune Diabetes (NIRAD) 4. *Diabetes Care*. 33(1):104–108.

Laugesen, E., Østergaard, J.A. & Leslie, R.D.G. 2015. Latent autoimmune diabetes of the adult: Current knowledge and uncertainty. *Diabetic Medicine*. 32(7):843–852.

Lauridsen, A.L., Vestergaard, P., Hermann, A.P., Brot, C., Heickendorff, L., Mosekilde, L. & Nexo, E. 2005. Plasma concentrations of 25-Hydroxy-Vitamin D and 1 , 25-Dihydroxy-Vitamin D are Related to the Phenotype of Gc (Vitamin D-Binding Protein): A Cross-sectional Study on 595 Early Postmenopausal Women. *Calcified tissue international*. 25(77):15–22.

Lee, P.G. & Halter, J.B. 2017. The Pathophysiology of Hyperglycemia in Older Adults : Clinical Considerations. *Diabetes Care*. 40(April):444–452.

Lips, P., Eekhoff, M., Schoor, N. Van, Oosterwerff, M., Jongh, R. De, Krul-poel, Y. & Simsek, S. 2016. Journal of Steroid Biochemistry & Molecular Biology Vitamin D and type 2 diabetes. *Journal of Steroid Biochemistry and Molecular Biology*. 173(2017):280–285.

Lombardi, G., Di Somma, C., Rubino, M., Faggiano, A., Vuolo, L., Guerra, E., Contaldi, P., Savastano, S., et al. 2011. The roles of parathyroid hormone in bone remodeling: prospects for novel therapeutics. *Journal of endocrinological investigation*. 34(7 Suppl):18–22. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/21985975 [2018, October 02].

Lu, L., Yu, Z., Pan, A., Hu, F.B., Franco, O.H., Li, H., Li, X., Yang, X., et al. 2009. Plasma 25-Hydroxyvitamin D Concentration and Metabolic Syndrome Among Middle-Aged and Elderly Chinese. *Diabetes care*. 32(7):1278–1283.

Mackawy, A.M.H. & Badawi, M.E.H. 2014. Association of vitamin D and vitamin D receptor gene polymorphisms with chronic in fl ammation , insulin resistance and metabolic syndrome components in type 2 diabetic Egyptian patients. *MGENE*. 2(2014):540–556.

Maeda, S.S., Saraiva, G.L., Kunii, I.S., Hayashi, L.F., Cendoroglo, M.S., Ramos, L.R. & Lazaretti-Castro, M. 2013. Factors affecting vitamin D status in different populations in the city of São Paulo, Brazil: The São PAulo vitamin D Evaluation Study (SPADES). *BMC Endocrine Disorders*. 13(14):1–8.

Magno, A.L., Ward, B.K. & Ratajczak, T. 2011. The calcium-sensing receptor: a molecular perspective. *Endocr Rev*. 32(1):3–30. [Online], Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20729338 [2019, February 04].

Malik, R.A., Mehta, P., Bhat, A.A., Farooq, R. & Shah, P.A. 2017. Vitamin D Receptor (VDR) Gene Polymorphism and Type 2 Diabeticmellitus (T2DM) -A Case Control Study in Ethnic Population of Kashmir Valley. *Advanced Research in Gastroenterology & Hepatology*. 4(5):10–15.

Martineau, A.R., Nhamoyebonde, S., Oni, T., Rangaka, M.X., Marais, S., Bangani, N., Tsekela, R., Bashe, L., et al. 2011. Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa. *Proceedings of the National Academy of Sciences*. 108(47):19013–19017.

Mathers, C.D. & Loncar, D. 2006. Projections of Global Mortality and Burden of Disease from 2002 to 2030. *PLoS Medicine*. 3(11):e442.

Matsha, T.E., Yako, Y.Y., Rensburg, M.A., Hassan, M.S., Kengne, A.P. & Erasmus, R.T. 2013. Chronic kidney diseases in mixed ancestry south African populations: Prevalence, determinants and concordance between kidney function estimators. *BMC Nephrology*. 14(1):1–10.

Maurya, R., Kumar, B. & Sundar, S. 2013. Evaluation of Salt-Out Method for the Isolation of Dna From Whole Blood: a Pathological Approach of Dna Based Diagnosis. *Int. J. LifeSc. Bt & Pharm. Res. Radheshyam Maurya et al*. 2(2).

McCollum, E. V & Davis, M. 1913. The necessity of certain lipids in the diet during growth. *J Biol Chem*. 15:167–75. [Online], Available: https://digitalcommons.ohsu.edu/cgi/viewcontent.cgi?article=1145&context=hca-cac [2018, September 29].

McCollum, E. V., Simmonds, N. & Becker, J.E. 1922. Studies on Experimental Rickets. An Experimental Demonstration of the Existance of a Vitamin which Promotes Calcium Deposition. *The American Society for Pharmacology and Experimental Therapeutics*. 53:293–312. [Online], Available: http://www.jbc.org/content/53/2/293.short [2018, September 29].

McKeown, N.M., Meigs, J.B., Liu, S., Saltzman, E., Wilson, P.W.F. & Jacques, P.F. 2004. Carbohydrate Nutrition, Insulin Resistance, and the Prevalence of the Metabolic Syndrome in the Framingham Offspring Cohort. *Diabetes Care*. 27(2):538–546.

Meigs, J.B., Cupples, L.A. & Wilson, P.W. 2000. Parental transmission of type 2 diabetes: the Framingham Offspring Study. *Diabetes*. 49(12):2201–7.

Mellanby, E. 1918. The part played by an '"accessory factor"' in the production of experimental rickets. *The Journal of Physiology*. 52:11–14. [Online], Available: https://digitalcommons.ohsu.edu/cgi/viewcontent.cgi?article=1269&context=hca-cac [2018, September 29].

Mohammadnejad, Z., Ghanbari, M., Ganjali, R. & Tavakkol, J. 2012. Association between vitamin D receptor gene polymorphisms and type 1 diabetes mellitus in Iranian population. 831–837.

Møller, U., Streym, S., Jensen, L., Mosekilde, L., Schoenmakers, I., Nigdikar, S. &

Rejnmark, L. 2013. Increased Plasma Concentrations of Vitamin D Metabolites and Vitamin D Binding Protein in Women Using Hormonal Contraceptives: A Cross-Sectional Study. *Nutrients*. 5(9):3470–3480.

Møller, U.K., Streym, S., Heickendorff, L., Mosekilde, L. & Rejnmark, L. 2012. Effects of 25OHD concentrations on chances of pregnancy and pregnancy outcomes: A cohort study in healthy Danish women. *European Journal of Clinical Nutrition*. 66(7):862–868.

Mooradian, A.D. 2009. Dyslipidemia in type 2 diabetes mellitus. *Nature Clinical Practice Endocrinology and Metabolism*. 5(3):150–159.

Moreira, T.S. & Hamadeh, M.J. 2010. The role of vitamin D deficiency in the pathogenesis of type 2 diabetes mellitus. e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism. 5(4):e155–e165.

Naik, R.G., Brooks-Worrell, B.M. & Palmer, J.P. 2009. Latent autoimmune diabetes in adults. *Journal of Clinical Endocrinology and Metabolism*. 94(12):4635–4644.

Naito, M., Miyaki, K., Naito, T., Zhang, L., Hoshi, K., Hara, A., Masaki, K., Tohyama, S., et al. 2007. Association between vitamin D receptor gene haplotypes and chronic periodontitis among Japanese men. *International Journal of Medical Sciences*. 4(4):216–222.

Narayan, K.M. V., Boyle, J.P., Geiss, L.S., Saaddine, J.B. & Thompson, T.J. 2006. Impact of Recent Increase in Incidence on Future Diabetes Burden: U.S., 2005-2050. *Diabetes Care*. 29(9):2114–2116.

Nguyen and El-Serag. 2010. The epidemiology of obesity. In Vol. 39 *Gastroenterology clinics*. 1–7.

Nguyen, N.T., Magno, C.P., Lane, K.T., Hinojosa, M.W. & Lane, J.S. 2008. Association of Hypertension, Diabetes, Dyslipidemia, and Metabolic Syndrome with Obesity: Findings from the National Health and Nutrition Examination Survey, 1999 to 2004. Journal of the American College of Surgeons. 207(6):928–934.

Nosratabadi, R., Arababadi, M.K., Salehabad, V.A., Shamsizadeh, A., Mahmoodi, M., Sayadi, A.R. & Kennedy, D. 2010. Polymorphisms within exon 9 but not

intron 8 of the vitamin D receptor are associated with the nephropathic complication of type-2 diabetes. *international journal of immunogenetics*. 37:493–497.

Ogunkolade, B.-W., Boucher, B.J., Prahl, J.M., Bustin, S. a, Burrin, J.M., Noonan, K., North, B. V, Mannan, N., et al. 2002. Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to vitamOgunkolade, B.-W., Boucher, B. J., Prahl, J. M., Bustin, S. a, Burrin, J. M., Noonan, K., … Hitman, G. a. (2002). Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to . *Diabetes*. 51(7):2294–300. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/12086963.

Ogurtsova, K., da Rocha Fernandes, J.D., Huang, Y., Linnenkamp, U., Guariguata, L., Cho, N.H., Cavan, D., Shaw, J.E., et al. 2017. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Research and Clinical Practice*. 128(2017):40–50.

Oh, J. & Barrett-connor, E. 2002. Association Between Vitamin D Receptor Polymorphism and Type 2 Diabetes or Metabolic Syndrome in Community-Dwelling Older Adults: The Rancho Bernardo Study. *metabolism*. 51(3):356–359.

Ortlepp, J.R., Metrikat, J., Albrecht, M., von Korff, A., Hanrath, P. & Hoffmann, R. 2003. The vitamin D receptor gene variant and physical activity predicts fasting glucose levels in healthy young men. *Diabetic medicine : a journal of the British Diabetic Association*. 20(6):451–4.

Ozfirat, Z. & Chowdhury, T.A. 2010. Vitamin D deficiency and type 2 diabetes. *Postgraduate Medical Journal*. 86(1011):18–25.

Palomer, X., González-Clemente, J.M., Blanco-Vaca, F. & Mauricio, D. 2008. Role of vitamin D in the pathogenesis of type 2 diabetes mellitus. *Diabetes, Obesity and Metabolism*. 10(3):185–197.

Parikh, S.J., Edelman, M., Uwaifo, G.I., Freedman, R.J., Semega-Janneh, M., Reynolds, J. & Yanovski, J. a. 2004. The relationship between obesity and serum 1,25-dihydroxy vitamin D concentrations in healthy adults. *The Journal of clinical endocrinology and metabolism*. 89(3):1196–9.

Pereira-Santos, M., Costa, P.R.F., Assis, A.M.O., Santos, C.A.S.T. & Santos, D.B. 2015. Obesity and vitamin D deficiency: a systematic review and meta-analysis. *Obesity Reviews*. 16(4):341–349.

Pittas, A.G., Lau, J., Hu, F.B. & Dawson-Hughes, B. 2007. The Role of Vitamin D and Calcium in Type 2 Diabetes. A Systematic Review and Meta-Analysis. *The Journal of Clinical Endocrinology & Metabolism*. 92(6):2017–2029.

Pittas, A.G., Sun, Q., Manson, J.E., Dawson-Hughes, B. & Hu, F.B. 2010. Plasma 25-hydroxyvitamin D concentration and risk of incident type 2 diabetes in women. *Diabetes Care*. 33(9):2021–2023.

Ponda, M.P., McGee, D. & Breslow, J.L. 2014. Vitamin D–Binding Protein Levels Do Not Influence The Effect of Vitamin D Repletion on Serum PTH and Calcium: Data From a Randomized, Controlled Trial. *The Journal of Clinical Endocrinology & Metabolism*. 99(7):2494–2499.

Poulsen, P., Kyvik, K.O., Vaag, A. & Beck-Nielsen, H. 1999. Heritability of type II (non-insulin-dependent) diabetes mellitus and abnormal glucose tolerance--a population-based twin study. *Diabetologia*. 42(2):139–45. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/10064092 [2018, September 27].

Powe, C.E., Ricciardi, C., Berg, A.H., Erdenesanaa, D., Collerone, G., Ankers, E., Wenger, J., Karumanchi, S.A., et al. 2011. Vitamin D-binding protein modifies the vitamin D-bone mineral density relationship. *Journal of Bone and Mineral Research*. 26(7):1609–1616.

Rahman, M.M., Hosen, M.B., Faruk, M.O., Hasan, M.M., Kabir, Y. & Howlader, M.Z.H. 2017. Association of vitamin D and vitamin D binding protein (DBP) gene polymorphism with susceptibility of type 2 diabetes mellitus in Bangladesh. *Gene*. 636(May):42–47.

Rana, J.S., Li, T.Y., Manson, J.E. & Hu, F.B. 2007. Adiposity compared with physical inactivity and risk of type 2 diabetes in women. *Diabetes care*. 30(1):53–8.

Rasmussen, S.S., Glümer, C., Sandbaek, A., Lauritzen, T. & Borch-Johnsen, K. 2008. Determinants of progression from impaired fasting glucose and impaired glucose tolerance to diabetes in a high-risk screened population: 3 year follow-

up in the ADDITION study, Denmark. *Diabetologia*. 51(2):249–257.

Reis, A.F., Hauache, O.M. & Velho, G. 2005. Vitamin D endocrine system and the genetic susceptibility to diabetes, obesity and vascular disease. A review of evidence. *Diabetes Metab*. 31(1262–3636 (Print)):318–325.

Riedemann, L., Lanvers, C., Deuster, D., Peters, U., Boos, J., Jürgens, H. & am Zehnhoff-Dinnesen, A. 2008. Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. *Pharmacogenomics Journal*. 8(1):23–28.

Ross, A.C., Manson, J.E., Abrams, S.A., Aloia, J.F., Brannon, P.M., Clinton, S.K., Durazo-arvizu, R.A., Gallagher, J.C., et al. 2011. The 2011 Report on Dietary Reference Intakes for Calcium and Vitamin D from the Institute of Medicine : What Clinicians Need to Know. *clinical endocrinology metabolism*. 96(January):53–58.

Saedisomeolia, A., Taheri, E., Djalali, M., Moghadam, A. & Qorbani, M. 2014. Association between serum level of vitamin D and lipid profiles in type 2 diabetic patients in Iran. *Journal of Diabetes & Metabolic Disorders*. 13(1):7.

Sahay, M. & Sahay, R. 2012. Rickets-vitamin D deficiency and dependency. *Indian journal of endocrinology and metabolism*. 16(2):164–76.

Saintonge, S., Bang, H. & Gerber, L.M. 2009. Implications of a New Definition of Vitamin D Deficiency in a Multiracial US Adolescent Population : *Paediatrics*. 123(3):797–803.

Schleithoff, S.S., Zittermann, A., Tenderich, G., Berthold, H.K., Stehle, P. & Koerfer, R. 2006. Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: a double-blind, randomized, placebo-controlled trial. The American Journal of Clinical Nutrition. 83(4):754–759.

Schuch, N.J., Garcia, V.C., Roberta, S., Ferreira, G. & Martini, L.A. 2013. Relationship between Vitamin D Receptor gene polymorphisms and the components of metabolic syndrome. *Nutrition journal*. 12(96):1–7.

Schwanstecher, C. & Schwanstecher, M. 2002. Nucleotide sensitivity of pancreatic ATP-sensitive potassium channels and type 2 diabetes. *Diabetes*. 51(SUPPL.

3):s358–s362.

Scragg, R., Sowers, M. & Bell, C. 2004. Serum 25-hydroxyvitamin D, diabetes, and ethnicity in the Third National Health and Nutrition Examination Survey. *Diabetes Care*. 27(12):2813–2818.

Shields, B.M., Hicks, S., Shepherd, M.H., Colclough, K., Hattersley, A.T. & Ellard, S. 2010. Maturity-onset diabetes of the young (MODY): how many cases are we missing? *Diabetologia*. 53(12):2504–2508.

Sibley, S.D., Thomas, W., De Boer, I., Brunzell, J.D. & Steffes, M.W. 2006. Gender and elevated albumin excretion in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) cohort: Role of central obesity. *American Journal of Kidney Diseases*. 47(2):223–232.

Singh, R., Shaw, J. & Zimmet, P. 2004. Epidemiology of childhood type 2 diabetes in the developing world. *Pediatr Diabetes*. 5(3):154–168.

Sinotte, M., Diorio, C. & Bérubé, S. 2009. Genetic polymorphisms of the vitamin D binding protein and plasma concentrations of 25-hydroxyvitamin D in premenopausal women. *The American journal …*. 25(3):634–640.

Sowers, J.R. & Epstein, M. 1995. Diabetes Mellitus and Associated Hypertension, Vascular Disease, and Nephropathy. *Hypertension*. 26(6):869–879.

Spanakis, E.K. & Golden, S.H. 2013. NIH Public Access. *Curr Diab Rep*. 13(6):1–18.

Speeckaert, M., Huang, G., Delanghe, J.R. & Taes, Y.E.C. 2006. Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. *Clinica Chimica Acta*. 372(1–2):33–42.

Spustová, V. & Dzúrik, R. 2004. [Vitamin D: synthesis, metabolism, regulation, and an assessment of its deficiency in patients with chronic renal disease]. *Vnitrni lekarstvi*. 50(7):537–43. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/15323262 [2019, February 04].

Sung, C.-C., Liao, M.-T., Lu, K.-C. & Wu, C.-C. 2012. Role of Vitamin D in Insulin Resistance. *Journal of Biomedicine and Biotechnology*. 2012(Figure 1):1–11.

Takiishi, T. & Gysemans, C. 2010. Vitamin D and Diabetes. *Endocrinology and Metabolism Clinics of NA*. 39(2):419–446.

Thorand, B., Zierer, A., Huth, C., Linseisen, J., Meisinger, C., Roden, M., Peters, A., Koenig, W., et al. 2011. Effect of serum 25-hydroxyvitamin D on risk for type 2 diabetes may be partially mediated by subclinical inflammation: Results from the MONICA/KORA Augsburg study. *Diabetes Care*. 34(10):2320–2322.

Tolonen, H., Kuulasmaa, K., Laatikainen, T. & Wolf, H. 2002. Recommendation for indicators, international collaboration, protocol and manual of operations for chronic disease risk factor surveys. *Project EHRM, European Health Risk Monitoring Project www-publications from the EHRM Project Helsinki, Finland: National Public Health Institute*.

Tripkovic, L., Lambert, H., Hart, K., Smith, C.P., Bucca, G., Penson, S., Chope, G., Berry, J., et al. 2012. Comparison of vitamin D 2 and vitamin D 3 supplementation in raising serum 25-hydroxyvitamin D status : a systematic review. *American Journal of Clinical Nutrition*. 95(August):1357–1364.

Uitterlinden, A.G., Fang, Y., Van Meurs, J.B.J., Van Leeuwen, H. & Pols, H.A.P. 2004. Vitamin D receptor gene polymorphisms in relation to Vitamin D related disease states. *Journal of Steroid Biochemistry and Molecular Biology*. 89–90(2004):187–193.

Unger, M.D., Cuppari, L., Titan, S.M., Magalhães, M.C.T., Sassaki, A.L., dos Reis, L.M., Jorgetti, V. & Moysés, R.M.A. 2010. Vitamin D status in a sunny country: Where has the sun gone? *Clinical Nutrition*. 29(6):784–788.

Valdivielso, J.M. & Fernandez, E. 2006. Vitamin D receptor polymorphisms and diseases. *Clinica Chimica Acta*. 371(1–2):1–12.

Vélayoudom-céphise, F., Larifla, L., Donnet, J., Maimaitiming, S. & Deloumeaux, J. 2011. Vitamin D deficiency , vitamin D receptor gene polymorphisms and cardiovascular risk factors in Caribbean patients with type 2 diabetes ☪. *Diabetes and Metabolism*. 37(6):540–545.

Vinodmahato, R., Gyawali, P., Raut, P.P., Regmi, P., Singh, K.P., Pandeya, D.R. & Gyawali, P. 2011. Association between glycaemic control and serum lipid profile

in type 2 diabetic patients: Glycated haemoglobin as a dual biomarker. *Biomedical Research*. 22(3):375–380. [Online], Available: http://www.alliedacademies.org/articles/association-between-glycaemic-control-and-serum-lipid-profile-in-type-2-diabetic-patients-glycated-haemoglobin-as-a-dual-biomarker.html [2018, October 03].

Wang, G., Li, Y., Li, L., Yu, F., Cui, L., Ba, Y., Li, W. & Wang, C. 2014. Association of the vitamin D binding protein polymorphisms with the risk of type 2 diabetes mellitus: a meta-analysis. *BMJ open*. 4(11):e005617.

Wang, Q., Xi, B. & Heather, K. 2012. Quantitative assessment of the associations between four polymorphisms (Fok I , Apa I , Bsm I , Taq I) of vitamin D receptor gene and risk of diabetes mellitus. *Mol bio Rep*. 39(2012):9405–9414.

Wang, T.J., Zhang, F., Richards, J.B., Kestenbaum, B., Van Meurs, J.B., Berry, D., Kiel, D.P., Streeten, E.A., et al. 2010. Common genetic determinants of vitamin D insufficiency: A genome-wide association study. *The Lancet*. 376(9736):180–188.

Wang, W., Ingles, S.A., Torres-Mejía, G., Stern, M.C., Stanczyk, F.Z., Schwartz, G.G., Nelson, D.O., Fejerman, L., et al. 2014. Genetic variants and non-genetic factors predict circulating vitamin D levels in Hispanic and non-Hispanic White women: The breast cancer health disparities study. *International Journal of Molecular Epidemiology and Genetics*. 5(1):31–46.

Ward, B.K., Magno, A.L., Walsh, J.P. & Ratajczak, T. 2012. The role of the calcium-sensing receptor in human disease. *Clinical Biochemistry*. 45(12):943–953.

Webb, a.r., kline, l. & holick, m.f. 1988. Influence of Season and Latitude on the Cutaneous Synthesis of Vitamin D $_3$: Exposure to Winter Sunlight in Boston and Edmonton Will Not Promote Vitamin D $_3$ Synthesis in Human Skin*. *The Journal of Clinical Endocrinology & Metabolism*. 67(2):373–378.

World Health Organization. 2016. Global Report on Diabetes. *Isbn*. 978:88.

Xu, H., GT, B., Q, Y., G, T., D, Y., C, C., J, S., A, N., et al. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. Journal of Clinical Investigation. 112(12):1821–1830.

Yogev, Y. & Hod, M. 2004. Epidemiology of gestational diabetes mellitus and its association with Type 2 diabetes. *Diabetic medicine : a journal of the British Diabetic Association*. 21:103–113.

Zella, J.B. & DeLuca, H.F. 2003. Vitamin D and autoimmune diabetes. *Journal of Cellular Biochemistry*. 88(2):216–222.

Zella, L.A., Shevde, N.K., Hollis, B.W., Cooke, N.E. & Pike, J.W. 2008. Vitamin D-binding protein influences total circulating levels of 1,25-dihydroxyvitamin D3 but does not directly modulate the bioactive levels of the hormone in vivo. *Endocrinology*. 149(7):3656–3667.

Zhang, C. & Ning, Y. 2011. Effect of dietary and lifestyle factors on the risk of gestational diabetes : *Am J Clin Nutr*. 94(supp1):1975–1979.

Zhang, J., Ye, J., Guo, G., Lan, Z., Li, X., Pan, Z., Rao, X., Zheng, Z., et al. 2016. Vitamin D Status Is Negatively Correlated with Insulin Resistance in Chinese Type 2 Diabetes.

Zittermann, A. 2006. Vitamin D and disease prevention with special reference to cardiovascular disease. *Prog Biophys Mol Biol*. 92(1):39–48.

Appendix 1: consent form

PARTICIPANT INFORMATION AND INFORMED CONSENT FORM FOR RESEARCH INVOLVING GENETIC STUDIES

TITLE OF RESEARCH PROJECT: PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA

REFERENCE NUMBER:

PRINCIPAL INVESTIGATORS:	**Professor Tandi Matsha (Cape Peninsula University of Technology)**
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Ethics approval:	**Cape Peninsula University of Technology Ethics Reference number:**
	CPUT/SW-REC 2015/H01
	University of Stellenbosch Ethics Reference number:
	N14/01/003

We would like to invite you to participate in a research study that involves genetic analysis and possible long-term storage of blood or tissue specimens. Please take some time to read the information presented here which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part initially.

This research study has been approved by the ethics Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: Ethics in Health Research: Principles, Structures and Processes.

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis.) Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

This research study seeks to address the increasing problem of diabetes and cardiovascular diseases such as heart attack and stroke amongst the mixed ancestry or coloured population of South Africa. In this study we shall identify people with diabetes and those at high risk of diabetes as well as investigate the environmental and genetic risk factors that predispose some individuals to the development of diabetes and cardiovascular diseases. Examples of environmental factors include body weight, diet, and physical activity. Additionally, this project aims to investigate whether oral health is a risk factor for diabetes and cardiovascular diseases. In this study we shall investigate whether some individuals have early cardiovascular diseases by using an ultrasound machine. This project also aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Our research team has previously conducted a similar research study involving the coloured community and found out that more that 18 out of 100 individuals had diabetes but did not know. We also found that some of the risk factors associated with diabetes in other populations were not necessary the same as those affecting the coloured population of South Africa. You have therefore been invited to take part in this research study to assist in establishing the risk factors for diabetes and cardiovascular diseases affecting the coloured people of South Africa.

A. You will be requested to provide information about your medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 30 minutes.

B. You shall be requested to provide a record of the medication you are currently taking, therefore if you are taking chronic medication, you shall be requested to provide this to the research team to record the medication.

C. Measurement such as weight, height, waist and hip will be done.

D. Fasting Venous Blood (20ml) will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood (10ml) will be collected. The blood will be used to determine whether you have diabetes or you are at high risk for developing diabetes.

E. The other tests that will be determined from your blood sample are: Cholesterol, triglycerides, creatine levels to assess your kidney function, liver enzymes to assess your liver, and biochemical markers for inflammation.

F. A finger prick blood sample (a drop of blood), to be taken at the same time of the first venous blood sample, may also be required from you. The finger prick blood sample will be used to test for diabetes or the risk of developing diabetes on a point-of-care test instrument. Researchers will compare the finger prick point-of-care diabetes test with that of the send away venous blood laboratory test and would be able to establish whether the point-of-care test provides the same accurate results as that of the laboratory. Point-of-care testing may in the future be used to provide fast and accurate results without the need to send blood away to a laboratory for processing. This may be of benefit to people undergoing testing for diabetes as results would be available within a few minutes.

G. The remainder of the blood sample will be used for genetic and future research studies. The serum and DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

H. For oral health, research study personnel will extract wooden toothpick, flocked brush, and mouthwash saliva samples from you to test for the presence of Porphyromonas gingivalis as an indicator for periodontal disease. Flocked brush and wood toothpick sampling will involve inserting devices in the subgingival crevice between the last upper premolar and the first upper molar. The device will sweep down the anterior surface of the first upper molar with the direction of motion away from the gum to minimize any potential discomfort. Mouthwash sampling will involve rinsing with 10 ml sterile saline solution for 20 seconds.

I. Early cardiovascular diseases will be performed by means of an ultrasound machine.

J. The research team will follow up on you on a yearly basis and some of these test may be repeated. The investigators wish to follow you up for your entire life. In the unfortunate event that you are deceased during the study period. The study team will review stats SA data and/or medical records to ascertain whether the cause of death was due to diabetes or cardiovascular diseases. . If you do not wish to be followed up on a yearly basis and your Statistics SA and/or medical records not to be accessed in the unfortunate event that you are deceased whilst being a participant in the study, you will have an opportunity to request that it be not accessed when you sign the consent form.

K. Radio imaging techniques will be done on consenting subjects. These include (i) ultra sound to assess whether you have signs of early cardiovascular diseases, (ii) computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases (iii) Dual-energy X-ray absorptiometry (DXA) devices will be used to study the morphology of the liver. These radio imaging techniques involve radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant. . If you do not wish to undergo any of these radio imaging techniques, you will have an opportunity to decline when you sign the consent form.

L. An eye examination will be done to test your eye vision and any other abnormalities inthe eye. For this examination, drops placed in your eyes widen (dilate) your pupils to allow the doctor to better view inside your eyes. The drops may cause your close vision to blur for a short while.

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse or doctor will be present on all occasions. You may also learn that you have diabetes, in which case you will be referred to your health care giver with the results for further treatment and management. If during the study it is discovered that you have changes in your genes that may lead to a serious disease, a genetic counsellor at the expense of the principal investigators will counsel you. Radio imaging techniques such as the CT-scan involves radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant.

Your personal results will be made known to you only if they indicate that you may:

- Have diabetes, thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.
- Have a condition or predisposition to developing diabetes that is treatable or avoidable

e.g. by a lifestyle modification.

- Need genetic counselling.

 However, participants with normal results who wish to know their results are free to contact the research team and their results will be given upon written request.

The blood samples may be stored indefinitely to accommodate new technologies that may develop. In the event that a technology is not available in South Africa to analyse your blood sample, your blood specimen may be sent to another country with the technology either now or at a later date. However, if your specimen is to be sent to another country, permission to do so will be sought from relevant bodies. Your blood specimen will be stored at the Cape Peninsula University of Technology.

Your blood will only be used for genetic research that is directly related to Diabetes and cardiovascular diseases. Also if the researchers wish to use your stored blood for additional research in this field they will be required to apply for permission to do so from the ethics Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

Your identity will be recorded once and kept confidential throughout. This is to allow the principal investigators to convey information that may be beneficial to you. Access will be limited to the principal investigators by assigning a special study code to all your data and blood samples. This means that your sample will be identified with a special study code that will remain linked to your name and contact details. However, during the entire research study, your blood specimens will be anonymised and the research staff won't be able to associate it with your name and contact details. You shall also be supplied this code so that if at anytime the investigators need to contact you, you may only identify yourself using your special code. Any scientific publications, lectures or reports resulting from the study will not identify you.

Some insurance companies may mistakenly assume that taking part in research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

You will not be paid to take part in this study although your out-of-pocket expenses may be reimbursed. The expenses that will be covered by the research team are those that include transportation to a hospital radiography department should you consent to radio imaging.

You should inform your family practitioner or usual doctor that you are taking part in a research study. You can contact

Prof T Matsha at 021 959 6366 or matshat@cput.ac.za,

If you have any further queries or encounter any problems, you can also contact the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee,

Chairperson: Prof Engel-hills at 0219596570 or EngelhillsP@cput.ac.za or

You will receive a copy of this information and consent form for your own records if it is requested. By signing below, I …….. agree to take part in a research project that includes genetic research study entitled (PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA).

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.

- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I have received a signed duplicate copy of this consent form for my records.

I agree that my blood or tissue sample can be stored indefinitely after the project is completed but that it is anonymised with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is anonymised, my rights to that sample are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

I agree that my blood or tissue sample can be stored indefinitely, but I can choose to request at any time that my stored sample be destroyed. My sample will be identified with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out.

OR

Please destroy my blood sample as soon as the current research project has been completed.

I consent that the research team may follow me up for yearly check-up AND in the unfortunate event that I am deceased whilst still part of the study, I consent that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

I do not consent to follow me up for yearly check-up BUT in the unfortunate event that I am deceased whilst still part of the study, I consent that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

I do not consent to follow me up for yearly check-up AND in the unfortunate event that I am deceased whilst still part of the study, I do not consent that the team accessing Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

□ I ***consent*** to ultra sound techniques to assess if I have early cardiovascular diseases

□ I ***do not consent*** to ultra sound techniques that assess if I have early cardiovascular diseases

AND

□ I ***consent*** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases

□ I ***do not consent*** to computed tomography scan (CT-scan) that accurately assess the fat content that is dangerous for cardiovascular diseases

AND

□ I **consent** to Dual-energy X-ray absorptiometry (DXA) used to study body composition.

□ I **do not consent** Dual-energy X-ray absorptiometry (DXA) used to study body composition

Signed at (place) ..... on (date) .....

Finger print

....

Signature of participant **Signature of witness**

I (name) ..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use a interpreter. (If a interpreter is used then the interpreter must sign the declaration below.

Signed at (place) ..... on (date) ..... 2016.

....

Signature of investigator **Signature of witness**

I (name) ..... declare that:

- I assisted the investigator (name) ..... to explain the information in this document to (name of participant) ..... Using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place) ..... on (date) ..... 2016.

....

Signature of interpreter **Signature of witness**