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The role of vitamin D in the aetiology of type 1 diabetes mellitus in the South African black population

Sureka Bhola

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

Johannesburg, 2018

Declaration

I Sureka Bhola declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Ms Sureka Bhola

On the _____day of _____2018

Dedication

I would like to dedicate this dissertation to my beloved Bhagwan Baba, my late dad Mr Devcharran Bhola and my mum Mrs Indubala Bhola.

Presentations arising from this study

Poster Presentations

Bhola S, Cave E, Prigge K, Bhana S, Crowther N, and Padoa C (2017)
"The FOK1 polymorphism in the vitamin D receptor gene is associated with type 1 diabetes in the black South African population" 23rd International Congress of Clinical Chemistry and Laboratory Medicine 2017 (Durban).

Bhola S, Cave E, Prigge K, Bhana S, Crowther N, and Padoa C (2017)
The FOK1 polymorphism in the vitamin D receptor gene is associated with type 1 diabetes in the black South African population. J. Endocrinol. Metab. Diab. S. Afr. Annual meeting of the Society of Endocrinology, Metabolism and Diabetes of South Africa, 2017 (Johannesburg).

Msibi DT, Cave E, **Bhola S**, Prigge K, Toman M, Crowther NJ and Padoa C (2017)
"The relationship between the vitamin D system and autoimmunity in African type 1 diabetic patients". 23rd International Congress of Clinical Chemistry and Laboratory Medicine 2017 (Durban).

Msibi DT, Cave E, **Bhola S**, Prigge K, Toman M, Crowther NJ and Padoa C (2016)
"The relationship between the vitamin D system and autoimmunity in African type 1 diabetic patients". Annual meeting of the Society of Endocrinology, Metabolism and Diabetes of South Africa, 2016 (Cape Town).

Abstract

Background: Type 1 diabetes (T1D) is a condition associated with the autoimmune mediated destruction of the pancreatic β-cells. Individuals who develop T1D often express autoantibodies (AAbs) which target β-cell epitopes (such as the 65kDa isoform of glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase related islet antigen 2 (IA-2) and Zinc transporter 8 (ZnT8)). Recently, vitamin D_3 has been shown to play an immunomodulatory role in T1D. Vitamin D_3 is formed by two successive hydroxylation steps in the liver and kidney by the enzymes encoded by the *CYP2R1* and *CYP27B1* genes, respectively. Vitamin D_3 exerts its effect through the vitamin D receptor (VDR), a ligand activated transcription factor. Activation of the VDR, through binding to vitamin D_3 mediates the suppression of pro-inflammatory cytokines which inhibit proliferation of Th1 cells. Th1 cells are responsible for the death of the β-cells. Four VDR single nucleotide polymorphisms (SNPs; BsmI [rs1544410], FokI [rs2228570], ApaI [rs7975232], TaqI [rs731236]) have been shown to be associated with the development of T1D however, these findings are not consistent. In addition, polymorphisms in the *CYP2R1* (rs10741657) and *CYP27B1* (rs10877012) genes have been shown to be associated with susceptibility to T1D. To our knowledge there is no data looking at these associations in the black South African population. This study, therefore, aimed to determine the prevalence of polymorphisms in the CYP and *VDR* genes and relate the allelic frequencies to serum levels of vitamin D_3 and T1D disease status in the South African black population. In addition, we aimed to determine GAD65, IA-2 and ZnT8 AAb positivity within the black South African population.

Methods: Clinically diagnosed black T1D patients (n=186) and non-diabetic black control participants (n=153) were recruited. All participants were genotyped for the four VDR SNPs and two CYP SNPs using PCR-RFLP. Vitamin D_3 was measured by HPLC using the ClinRep High Performance Liquid Chromatography. GAD65, IA-2 and ZnT8 AAbs were measured by ELISA and glucose was measured on the Advia Chemistry System.

Results: In our cohort the mean age at diagnosis was 20.7 ± 8.4 years with a median duration of 7 [2; 11] years. The *VDR* and *CYP* gene SNPs were not associated with T1D in the South African black population. Similarly, low levels of vitamin D_3 were not associated with the disease. Multiple regression analysis showed that vitamin D_3

levels were 5.34 nmol/L lower in individuals carrying the FokI CC genotype compared to individuals with CT/TT genotypes (p = 0.021). In addition, an increasing number of *VDR* risk alleles were associated with lower vitamin D_3 levels (0 risk alleles: 66.53 ± 23.00 nmol/L vs. 8 risk alleles: 47.30 ± 14.87 nmol/L; p = 0.006). In our T1D patients 55.6% were AAb positive (GAD65 = 51%, IA-2 = 12.9% and ZnT8 = 17.6%). IA-2 and ZnT8 AAb positivity was associated with a significantly younger age at diagnosis (16.40 ± 5.53 vs. 21.62 ± 8.56 years; p < 0.001 and 17.00 ± 6.49 vs. 21.48 ± 8.50 years; p = 0.006 respectively) and shorter duration of disease (p = 0.032 and p = 0.008, respectively). GAD65 positive individuals had a 23-fold higher risk of developing T1D (p < 0.001).

Conclusion: We found no association between the *VDR* and *CYP* gene polymorphisms in the black South African population. However, we did find that the FokI CC genotype was associated with lower levels of vitamin D_3. Whilst AAbs are markers of T1D, AAb negativity is not sufficient to exclude a T1D diagnosis in our cohort. AAb positive patients were more likely to develop T1D at an earlier age than those who were AAb negative. The South African black population has an average age at diagnosis a decade later than that seen in white populations. In addition, the frequency of AAbs was lower than that observed in white populations. These findings suggest South African black T1D patients have a unique disease aetiology.

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List of abbreviations and symbols

ADA	American Diabetes Association
APC	Antigen presenting cell
AAb	Autoantibody
ATP	Adenosine triphosphate
bp	Base pairs
β-cell	Beta cell
B cells	B lymphocytes
BMI	Body Mass Index
Ca^{2+}	Calcium
CD	Cluster of differentiation
CD4+	Cytotoxic T cells (4)
CD8+	Cytotoxic T cells (8)
CHBAH	Chris Hani Baragwanath Academic Hospital
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CTLA-4	Cytotoxic T lymphocyte associated-4
CYP2R1	Cytochrome P450 Family 2 subfamily R member 1
CYP24A1	Cytochrome P450 family 24 subfamily A member 1
CYP27B1	Cytochrome P450 family 27 subfamily B member 1
DM	Diabetes mellitus
DBP	Vitamin D binding protein
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diaminetetra acetic acid
GAD65	65kDa isoform of glutamic acid decarboxylase
H_2SO_4	Sulphuric acid
HbA1c	Glycosylated haemoglobin A 1 c
HLA	Human leukocyte antigen
HPLC	High Performance Liquid Chromatography
ICA	Islet cell cytoplasmic antibodies
IA-2	Protein tyrosine phosphatase related islet antigen 2
IAA	Insulin autoantibodies
IFNγ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
IL-1β	Interleukin 1 beta
IL2RA	Interleukin-2 receptor subunit alpha
IS	Internal standard
IU	International units
M	Molar
$MgCl_2$	Magnesium chloride
MHC	Histocompatibility complex
Min	Minutes
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenosine dinucleotide
NADH	Nicotinamide adenosine dinucleotide hydrogenase
NFκB	Nuclear factor kappa B
NFAT	Nuclear factor of activated T cells
PCR	Polymerase chain reaction
PTPN22	Protein tyrosine phosphatase non receptor type 22
PTH	Parathyroid hormone
RFLP	Restriction fragment length polymorphism
RXR	Retinoid X receptor
SANBS	South African Blood Transfusion Services
Secs	Seconds
SSA	Sub-Saharan Africa
SST	Serum-separating tubes
TBE	Tris – Borate - EDTA
T cells	T lymphocytes
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGFβ	Transforming growth factor β
Th0	Naïve T cells
Th1	T helper 1
Th2	T helper 2
TNFα	Tumour necrosis factor alpha
TNFAIP3	Tumour necrosis factor-alpha–induced protein 3

UVB	Ultra violet B
VDR	Vitamin D receptor
VDRE	Vitamin D response elements
vs.	Versus
WHR	Waist to hip ratio
ZnT8	Zinc transporter 8
$1,25(OH)_2D_3$	1,25 hydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
$1,24(OH)_2D_3$	1,24-dihydroxyvitamin D_3
$24,25(OH)_2D_3$	24,25-dihydroxyvitamin D_3
g	Relative centrifugal force
kb	Kilobases
kg/m^2	Kilogram per metre squared
ml	Millilitres
mmol/L	Millimole per litre
nmol/L	Nanomoles per litre
n	Number
pmol	Picomole
rpm	Revolutions per minute
µl	Microlitres

Chapter 1

1 Literature Review

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) has been defined by the American Diabetes Association (ADA) as a "collection of metabolic diseases characterised by hyperglycaemia as a consequence of defects in insulin secretion, insulin action or both" (American Diabetes Association, 2004).

Symptoms of DM include polyuria, polydipsia, weight loss and polyphagia. Prolonged hyperglycaemia often results in micro- and macro-vascular complications, namely; nephropathy, peripheral and autonomic neuropathy, retinopathy, sexual dysfunction and cardiovascular disease (American Diabetes Association, 2013).

DM is not a single homogenous condition and is subdivided into four broad categories based on the pathogenesis of the disease namely; type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes and other specific types (Table 1.1).

Table 1.1: Aetiological classification of diabetes mellitus*

CATEGORY	AETIOLOGICAL CLASSIFICATION
Type 1 diabetes	Immune mediated or idiopathic β-cell destruction, usually leading to absolute insulin deficiency
Type 2 diabetes	May range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance
Other specific types	Genetic defects of β-cell function and insulin action, diseases of the exocrine pancreas, endocrinopathies, chemical induced, infections, uncommon forms of immune-mediated diabetes and genetic syndromes
Gestational diabetes	Diabetes diagnosed during pregnancy

* Adapted from (American Diabetes Association, 2012)

1.2 Type 1 diabetes

T1D is characterised by an autoimmune response which results in the destruction of the insulin secreting β-cells of the islets of Langerhans, such that at the time of diagnosis, only approximately 20% of the functional β-cell mass remains (Foulis et al., 1986). T1D is considered to be the second most common chronic disease of childhood with a peak incidence observed in children between the ages of 10-14 years (Atkinson et al., 2014). However, it has recently been reported that the greatest increase in incidence occurs in children less than five years of age (Simmons and Michels, 2015). As of 2014 it was estimated that between 29.3 and 38.7 million individuals worldwide suffer from T1D (You and Henneberg, 2016). Globally the incidence of T1D is increasing at a rate of approximately 3% annually (I. D. F. Diabetes Atlas Group, 2015). The incidence of T1D varies widely geographically, with northern countries such as Finland and Sardinia having the highest incidence rates worldwide (42.9/100 000 inhabitants per year) (Verkauskiene et al., 2016, Atkinson et al., 2014, Atkinson and Eisenbarth, 2001) while China and Venezuela have the lowest incidence rates (0.1/100 000 cases per year) (Verkauskiene et al., 2016, Noble and Valdes, 2011).

The incidence of T1D also varies with ethnicity. White populations have the highest incidence of the disease, followed by Black populations. The lowest incidence has been observed in Asian populations (Raha et al., 2009).

1.2.1 Pathogenesis of T1D

The exact mechanism of β-cell death (Figure 1.1) in T1D is not fully understood. Damage to β-cells (potentially triggered by environmental factors) results in the release of β-cell specific autoantigens. The autoantigens are taken up by antigen presenting cells (APC; macrophages, dendritic cells or B cells) and presented to naïve CD4+ T-helper (Th)0 cells in the pancreatic lymph nodes (Ergun-Longmire and Maclaren, 2000). This stimulates the APCs to secrete interleukin (IL)-12 causing naïve CD4+ Th0 cells to differentiate into Th1 cells (pro-inflammatory) (Vaseghi and Jadali, 2016). T lymphocytes are a major source of cytokines and are considered to be the hormonal messenger of the immune system as they are responsible for most of the biological effects of the immune system (Harinarayan, 2014, Prietl et al., 2013).

The Th1 cells then secrete the pro-inflammatory cytokines, IFNγ and IL-2 (Ergun-Longmire and Maclaren, 2000). IFNγ stimulates resting macrophages to release toxic cytokines (IL-1β, tumour necrosis factor alpha (TNFα)) and free radicals which cause β-cell damage (Ergun-Longmire and Maclaren, 2000, Azar et al., 1999, Tisch and McDevitt, 1996). IL-2 stimulates the migration of CD8+ cells to the inflamed islets where they differentiate into CD8+ cytotoxic T cells upon dendritic cell presentation of autoantigens. The cytotoxic T cells then release perforin (pore-forming glycoprotein), granzymes and superoxides in combination with Fas mediated apoptosis of β-cells (Kukreja and Maclaren, 1999). The perforin polymerises and binds to the target cell's plasma membrane in the presence of Ca^{2+} ions to form pores in the β-cell. The pores allow for the passive diffusion of granzymes into the cell (Osinska et al., 2014). Granzymes are serine proteases that induce apoptosis by activating caspases 3 and 7, cleaving many substrates, including caspase-activated DNase, to execute target cell (β-cell) death (Cullen et al., 2010).

Destruction of the β-cells can be prevented by the secretion of IL-4 by natural killer cells at the beginning of Th0 differentiation. The IL-4 stimulates naïve CD4+ Th0 cells to differentiate preferentially into Th2 cells (anti-inflammatory). Th2 cells secrete IL-4 and IL-10 which prevent Th1 proliferation and IFNγ induced macrophage activation resulting in β-cell survival (Vaseghi and Jadali, 2016, Azar et al., 1999, Liblau et al., 1995).

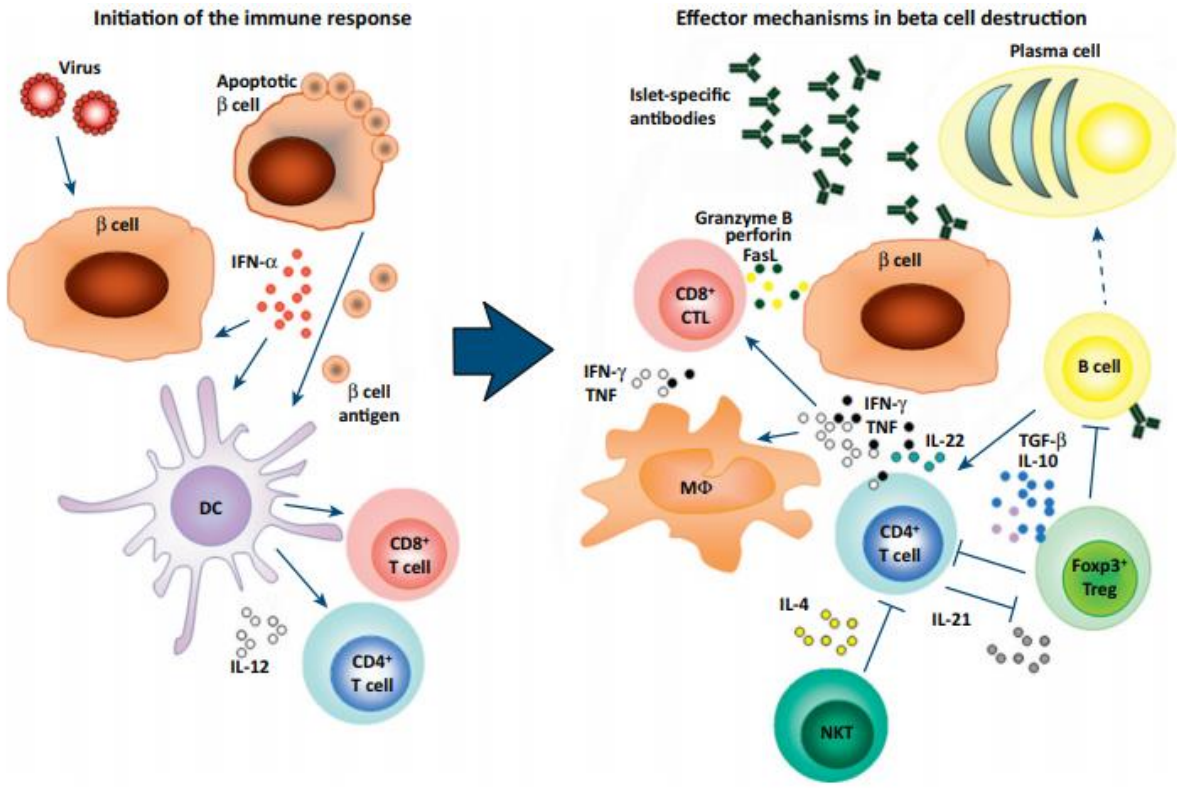


Figure 1.1: The role of the Th1 pathway in the destruction of pancreatic β-cells

Environmental factors such as viral infections or inflammatory processes may lead to β-cell death. β-cell antigens are released by β-cells undergoing secondary necrosis, activating the APCs. Naïve CD4+ Th0 cells in the pancreatic lymph nodes differentiate into Th1 cells on presentation of the islet antigens. The Th1 cells release pro-inflammatory cytokines (IFNγ and IL-2). These inflammatory cytokines activate macrophages and CD8+ cytotoxic cells to release toxic cytokines (IL-1β and TNFα), and apoptosis inducing proteins such as Fas leading to destruction of the β-cell (Wallberg and Cooke, 2013).

1.2.2 Autoantibodies in T1D

Autoantibodies (AAbs) are generated by the body when it fails to recognise epitopes as 'self', resulting in an immune response targeted to the body (Kukreja and Maclaren, 1999, Pihoker et al., 2005). In T1D AAbs are directed to several β-cell antigens. The first AAbs identified to have an association with the development of T1D were islet cell cytoplasmic antibodies (ICA) (Bottazzo et al., 1974, MacCuish et al., 1974). Subsequently, AAbs to insulin (IAA; Palmer et al. (1983)), the 65kDa isoform of glutamic acid decarboxylase (GAD65; Baekkeskov et al. (1982)), protein tyrosine phosphatase related islet antigen 2 (IA-2; Passini et al. (1995)) and more recently zinc transporter 8 (ZnT8; Wenzlau et al. (2007)) have been described.

Islet AAb development can occur years before the clinical diagnosis of T1D (Simmons and Michels, 2015, Tiberti et al., 2011). More than 90% of newly diagnosed T1D patients have one or more of these AAbs (Atkinson et al., 2014, Simmons and Michels, 2015). Individuals presenting with two or more AAbs have a 39% risk of developing diabetes within 3 years and a 69% risk within 5 years (Verge et al., 1998, Jasinski and Eisenbarth, 2005). In addition, the earlier the AAbs appear in children, the more likely and rapidly they are to progress to T1D (Jasinski and Eisenbarth, 2005). Detection of these AAbs provides a valuable marker in the prediction, diagnosis and monitoring of T1D (Tiberti et al., 2011). However, the levels of AAbs are influenced by the age of the patient, duration of disease and ethnicity (Fida et al., 2001, Wenzlau et al., 2007). In addition, AAb titres might vary depending upon which method was used for quantitation (Bingley et al., 2010, Bonifacio et al., 2010).

1.2.2.1 Insulin AAbs

IAA are generally the first AAbs to develop in children and persistently high levels lead to 100% advancement to T1D (Simmons and Michels, 2015, Atkinson et al., 2014, Nokoff and Rewers, 2013). IAA are usually associated with appearance at a young age; 35 - 60% of newly diagnosed children were IAA positive compared to only 20% of newly diagnosed adults (Sabbah et al., 2000, Winter et al., 2002). These AAbs are found to be associated with HLA DR4 and subsequent progression to multiple AAb positivity (Achenbach et al., 2004, Winter et al., 2002, Steck et al., 2011, Simmons and Michels, 2015). The measurement of insulin AAbs is of no use after initiation of insulin therapy (Fineberg et al., 2007).

1.2.2.2 Glutamic acid decarboxylase 65 AAbs

GAD65 AAbs are found in greater than 70% of patients with T1D (Pihoker et al., 2005) (Baekkeskov et al., 1990). GAD65 AAbs are the most persistent of the T1D AAbs. However, some studies have shown a decline of these AAbs over time (Atkinson and Eisenbarth, 2001, Rodacki et al., 2004). Individuals with the HLA DR3 allele have a greater chance of being GAD65 AAb positive (Stayoussef et al., 2011).

1.2.2.3 Protein tyrosine phosphatase related islet antigen 2 AAbs

IA-2 AAbs are detected in 60 – 70% of all T1D patients and in 85-90% of patients at the time of diagnosis (Raha et al., 2009). IA-2 AAbs are more prevalent in patients that develop T1D at a younger age and are associated with rapid disease progression. The frequency of these AAbs tends to decline with increasing duration of disease (Simmons and Michels, 2015). IA-2 AAb positivity is associated with the presence of the HLA-DR4 allele (Simmons and Michels, 2015, Raha et al., 2009).

1.2.2.4 ZnT8 AAbs

ZnT8 AAbs are found in 60-80% of newly diagnosed TID patients (Fakhfakh, 2011). Interestingly, ZnT8 AAb positivity has been noted in T1D patients who did not express the more common IA-2 and GAD65 AAbs (Fakhfakh, 2011). As seen with IA-2 AAbs, the frequency of ZnT8 AAbs decline following diagnosis (Wenzlau et al., 2015). ZnT8 AAbs seldom present before the age of three years (Knip and Siljander, 2008). ZnT8 AAbs decline rapidly with increasing duration of disease (Vaziri-Sani et al., 2010, Howson et al., 2012)

1.2.3 The aetiology of T1D

T1D is a multifactorial disease caused by a combination of environmental and genetic factors (Simmons and Michels, 2015, Paschou et al., 2018).

1.2.3.1 The genetics of T1D

Numerous genes have been associated with the development of T1D. The human leukocyte antigen (HLA) region confers approximately 50% of the inherited risk for the disease (Pociot and McDermott, 2002). The HLA region is a cluster of more than 200 genes found within the major histocompatibility complex (MHC) on chromosome 6p21.31 and spans a region of approximately 3.5 megabases (Noble and Erlich, 2012, Raha et al., 2009). The HLA class II genes (HLA-DR and HLA-DQ) are the main determinants of T1D risk (Atkinson et al., 2014, Noble and Valdes, 2011). Studies have shown that DRB1*0301-DQA1*0501-DQB1*0201 and DRB1*0401-DQA1*0301-DQB1*0302 are the main susceptibility haplotypes in T1D (Lie et al., 1999, Pociot and McDermott, 2002). The

predominant protective HLA haplotype is DRB*1501-DQA1*0102-DQB1*0602 (Kantarova and Buc, 2007).

In addition to the HLA region, there are more than 50 non-HLA genes that significantly affect the risk for T1D (Pociot et al., 2010). The major non-HLA genes include the insulin gene, cytotoxic T lymphocyte associated-4 gene (CTLA-4), protein tyrosine phosphatase non receptor type 22 gene (PTPN22) and the interleukin 2 receptor alpha gene (IL2RA/CD25) (Raha et al., 2009, Roep et al., 2016, Atkinson, 2012, Paschou et al., 2018). These susceptibility genes play a role in immune responsiveness and/or immune regulation (Figure1. 2) (Atkinson, 2012).

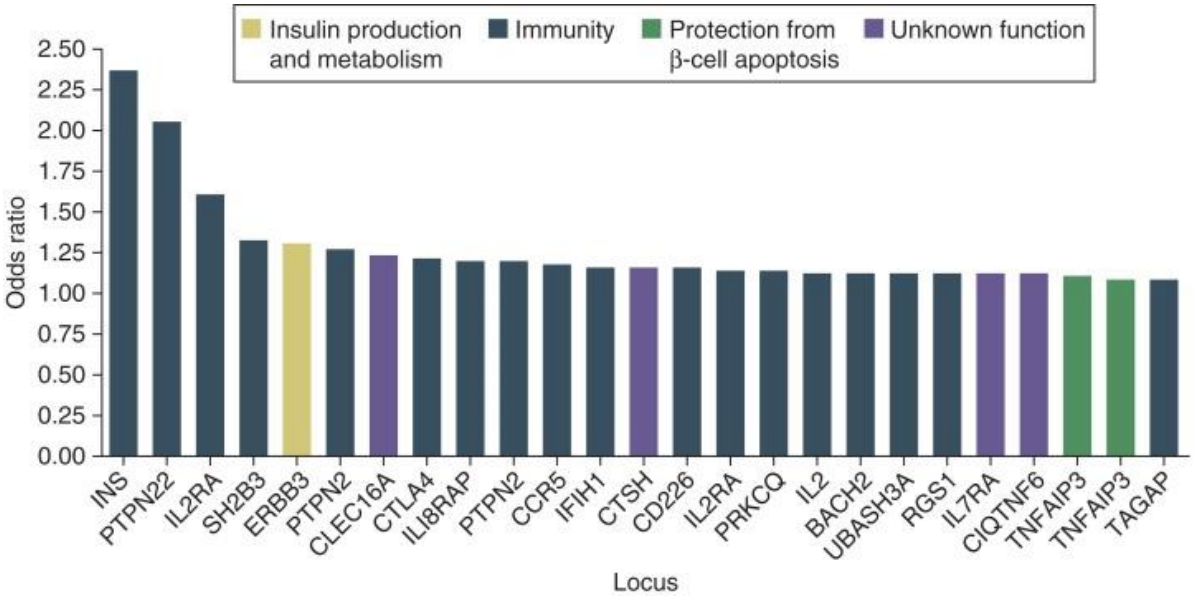


Figure 1.2: Non-HLA loci associated with an increased risk of T1D development

The X axis represents the possible candidate genes associated with T1D while the Y axis represents the odds ratio. The bars illustrating the odds ratio have been colour coded suggesting the probable role of these loci in T1D susceptibility. IL2RA and TNFAIP3 have been shown twice on the graph as there has been evidence that these two loci have two independent effects on risk of the disease and with different odds ratios (Atkinson, 2012).

The likelihood of developing T1D in the general population is 1:300. The risk is increased to 1:7 in children who have a genetically related sibling diagnosed with T1D. The probability of children with T1D mothers acquiring diabetes is 3% and with T1D fathers the risk increases to 5% (Simmons and Michels, 2015). The identical twin of a T1D patient has a 70% risk of developing the disease. If T1D in one of the identical twins occurs before the age of five years, the risk of disease development in the second twin increases

by more than 50%. On the other hand, if the disease manifests after the age of 25, the risk to the second twin is less than 10% (Michels and Gottlieb, 2000). The studies looking at identical twins clearly illustrate that genetics is not the only factor involved in developing T1D and suggests that environmental factors also need to be studied.

1.2.3.2 Environmental factors

Several environmental factors have been shown to contribute to β-cell destruction. These factors include viral infections, toxins, vaccines, dietary intake, stress and climatic conditions (Roep et al., 2016, Rewers and Ludvigsson, 2016, Raha et al., 2009, Penna-Martinez and Badenhoop, 2017). These elements have been thought to act as a trigger in genetically susceptible individuals initiating an autoimmune response (appearance of AAbs), insulitis and ultimately destruction of the β-cells (Raha et al., 2009, Penna-Martinez and Badenhoop, 2017).

1.2.3.2.1 Viral infections

Exposure to certain infectious material and viral infections (enterovirus, rubella, mumps, rotavirus, cytomegalovirus and cocksackie virus) precipitate an autoimmune response (Eisenbarth, 1986, Raha et al., 2009). It is hypothesised that certain viruses mimic the protein sequence of GAD65 expressed in the pancreatic β-cells. This causes the immune system to erroneously destroy the β-cells (Simmons and Michels, 2015).

1.2.3.2.2 Cow's milk

The introduction of infants to cow's milk may potentially initiate T1D in genetically susceptible individuals (Raha et al., 2009). It is believed that consuming cow's milk results in higher levels of bovine insulin antibodies. Due to the similarities of bovine and human insulin, the antibodies raised to bovine insulin cross react with human insulin. Similarly, some studies have shown that the consumption of cow's milk may increase the development of anti-islet AAbs (Rewers and Ludvigsson, 2016, Raha et al., 2009, Michels and Gottlieb, 2000). Interestingly, breast feeding infants has been shown to have a protective effect against the development of T1D. This protective effect is believed to be due to the presence of immunoglobulin (Ig) A, immune cells and a variety of hormones that prevent infection and perhaps autoimmunity (Egro, 2013).

1.2.3.2.3 Geographical location and seasonal changes

Epidemiological studies have shown large geographical variation in the incidence of T1D, with higher degrees of latitude (i.e. further north from the equator) correlating with a higher incidence of the disease (Mutlu et al., 2011, Mohr et al., 2008, Liu et al., 2015, Grammatiki et al., 2018). The higher incidence of T1D in the northern European populations could be related to vitamin D insufficiency (due to decreased sun exposure) (Newhook et al., 2012).

Interestingly, seasonal patterns of T1D onset have also been observed with the majority of new cases presenting in the autumn/winter months (Greer et al., 2013, Atkinson et al., 2014). Offspring born during spring tend to be at an increased risk of developing T1D potentially due to the final trimester being in the winter months and hence lower maternal vitamin D levels (Simmons and Michels, 2015). These findings, showing an inverse correlation between sun exposure and incidence of disease, point to a role for vitamin D in the pathogenesis of T1D (Liu et al., 2015).

1.3 Vitamin D

Vitamin D is a fat soluble steroid hormone and has two major forms, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) (Kamen and Tangpricha, 2010, Guo et al., 2006, Liu et al., 2015, Paschou et al., 2018). The main sources of vitamin D are dietary intake and synthesis within the skin (Sung et al., 2012).

1.3.1 Dietary vitamin D

Dietary vitamin D differs depending on which food source it is derived from. Vitamin D_2 originates from plants, vegetable sources, fungi, eggs, certain types of yeast and fortified foods such as orange juice, milk and cereal. Vitamin D_3 is found in foods such as fatty fish (cod, sardines and salmon) (Harinarayan, 2014, Prietl et al., 2013, Zhang and Naughton, 2010). Dietary intake accounts for a small proportion of vitamin D_3 which is absorbed by the intestines (Prietl et al., 2013, Guo et al., 2006, Kamen and Tangpricha, 2010). The main source of vitamin D_3 is understood to be exposure to sunlight and absorption through the skin (Chakhtoura and Azar, 2013, Mathieu and Badenhoop, 2005, Seshadri, 2011).

1.3.2 Vitamin D synthesis and metabolism

Vitamin D in its natural form is biologically inactive and has to be metabolised to become active (Figure 1.3) (Seshadri, 2011). Upon skin exposure to ultraviolet B (UVB) light, 7-dehydrocholesterol is converted to vitamin D_3 (Rose et al., 2013). Vitamin D_3 is hydroxylated in the liver by 25-hydoxylase (encoded by the *CYP2R1* gene) forming 25-hydroxyvitamin D_3 [25(OH)D_3] which is biologically inert. The 25(OH)D_3 is further hydroxylated by the kidney 1-alpha hydroxylase (1α-OHase; encoded by the *CYP27B1* gene) forming the biologically active 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$] (Kamen and Tangpricha, 2010, Kongsbak et al., 2013, Arnson et al., 2007). This action is regulated by calcium and hormones such as parathyroid hormone (PTH), calcitonin, growth hormone and insulin like growth factor. The production of PTH is prompted by low levels of calcium stimulating CYP27B1 activity resulting in 1,25(OH)$_2D_3$ production. Once the level of calcium normalises, CYP27B1 activity ceases (Prietl et al., 2013, Penna-Martinez and Badenhoop, 2017).

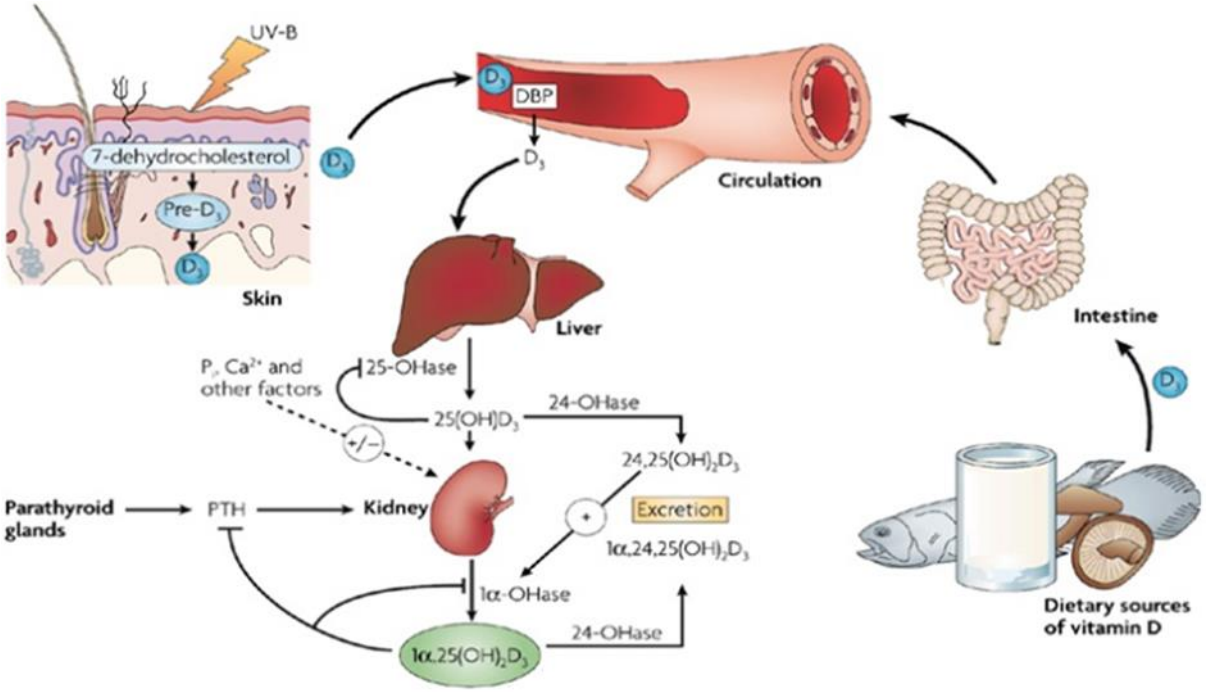


Figure 1.3: Schematic representation of vitamin D metabolism

Vitamin D is obtained from the diet, supplements and exposure to sunlight. Upon exposure to UVB, 7-dehydrocholesterol is converted to the biologically active 1,25(OH)$_2D_3$ via two hydroxylation steps in the liver and kidney. The main effects of 1α,25(OH)$_2D_3$ on the various target tissues such as intestines, bone, tumour micro environment and immune cells are highlighted above (Deeb et al., 2007).

Both 25(OH)D_3 and 1,25$(OH)_2D_3$ can be further hydroxylated to their less active metabolites 1,24-dihydroxyvitamin D_3 [1,24-$(OH)_2D_3$] and 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] by 24-hydroxylase (encoded by the *CYP24A1* gene). These metabolites are degraded and eventually excreted in bile, thereby limiting excess concentrations of 25(OH)D_3 and 1,25$(OH)_2D_3$ (Prietl et al., 2013, Penna-Martinez and Badenhoop, 2017).

1.3.3 The association of vitamin D with T1D

Several studies, in different population groups, have shown a correlation between low levels of vitamin D_3 and risk of T1D (Chakhtoura and Azar, 2013, Bin-Abbas et al., 2011, de Oliveira et al., 2018). Vitamin D_3 deficiency during pregnancy is associated with infants at increased risk of developing T1D (Greer et al., 2013, Dong et al., 2013). Supplementation of infants with vitamin D significantly reduced their risk of T1D development (pooled odds ratio 0.71, 95% CI 0.60 to 0.84), and the higher the vitamin D concentration the lower their risk of developing T1D (Kamen and Tangpricha, 2010, Grammatiki et al., 2018). In a cross sectional study carried out on Italian T1D children, adolescents and immigrants, an overall insufficiency of 25(OH)D_3 was seen and T1D patients with levels below 25 nmol/L needed higher doses of insulin therapy (Savastio et al., 2016). In addition, the risk of patients presenting with diabetic ketoacidosis (DKA) was directly proportional to lower levels of 25(OH)D_3 (Savastio et al., 2016, Mathieu et al., 1995, Hypponen et al., 2001). A birth cohort study conducted in northern Finland found that the babies vitamin D supplementation (2000 IU/day) showed protective effects by restoring insulin secretion due to its immunomodulatory effects and thereby improving their diabetes status (Hypponen et al., 2001). Similarly, children receiving vitamin D supplementation (2000 IU/day) in the first 12 months after birth were less likely to develop TID (Seshadri, 2011). In a study by Dong and colleagues, it was established that supplementation of vitamin D during the early stages of life was linked to a reduced risk of T1D (Dong et al., 2013).

In Middle Eastern countries, such as Kuwait and Saudi Arabia, a higher incidence of vitamin D deficiency is observed despite the high temperatures and close proximity to the equator. This may be due to people staying indoors due to high temperatures, wearing of traditional clothing that leaves very little skin exposed to sunlight and social customs like young adults avoiding sunlight (Rasoul et al., 2016, Al-Daghri et al., 2014). Interestingly,

there is a higher prevalence of T1D in these countries thought to be due to the high levels of vitamin D deficiency (70-90%) (Al-Daghri et al., 2014, Lips, 2007).

Numerous studies have indicated that vitamin D_3 may be protective against the development of T1D; however the levels of vitamin D_3 thought to provide the protective effect have not been standardised (Altieri *et al*., 2017, Mathieu and Badenhoop, 2005). The true indicator of vitamin D_3 status is through the measurement of the active metabolite, 25(OH)D_3, with a half-life of approximately two weeks (Hart, 2012). In general, between 400 IU/day and 2000 IU/day are recommended by the Institute of Medicine and the Endocrine Society in the USA depending on factors such as age, gender, body weight, pregnancy and levels of deficiency or insufficiency (Pludowski et al., 2018, Holick et al., 2011).

1.3.4 Vitamin D as an immune modulator

Traditionally, vitamin D has been associated with calcium and phosphate homeostasis and regulation of bone metabolism (Ogunkolade et al., 2002, Kamen and Tangpricha, 2010, Yang et al., 2013). However, vitamin D has more recently been shown to display many roles in biological processes within the target cells of the immune system (Yang et al., 2013, Mathieu and Badenhoop, 2005, de Oliveira et al., 2018).

Vitamin D_3 functions predominantly through activating the vitamin D receptor (VDR). The VDR is a ligand activated transcription factor expressed in numerous cells, including T cells, B cells, APCs, immune cells and Pancreatic β-cells (Mukhtar et al., 2017, O'Neill et al., 2013) It is, thus, not surprising that activation of the VDR results in transcriptional regulation of a number of genes such as those involved in cellular proliferation and differentiation and immune response (O'Neill et al., 2013, Arnson et al., 2007, Kamen and Tangpricha, 2010).

All circulating metabolites of vitamin D_3 cross the plasma membrane bound to a carrier protein, vitamin D binding protein (DBP) (Cooke et al., 1988, Mathieu and Badenhoop, 2005). DBP is a single chain serum glycoprotein synthesised and secreted by the liver. It is polymorphic, forms a complex with vitamin D_3 and delivers it to the target tissue where vitamin D exerts its effects (Mathieu and Badenhoop, 2005, Sung et al., 2012). The exact mechanisms of VDR-mediated immune modulation are not fully understood. It is thought that once 1,25$(OH)_2D_3$ enters the cell, it binds the VDR inducing a conformational change

(Figure 1.4) (Baeke et al., 2010, Kongsbak et al., 2013). This change allows for retinoid X receptor (RXR)-VDR heterodimerisation. The 1,25$(OH)_2D_3$-RXR-VDR complex then crosses the nuclear membrane and enters the nucleus. The complex then binds to specific DNA sequence elements i.e. vitamin D response elements (VDRE) located in vitamin D responsive genes. This leads to activation or suppression of transcription depending on the type of co-regulatory factor present. Within T cells, the vitamin D signalling pathway down regulates the expression of IFNγ through direct interaction of 1,25$(OH)_2D_3$-RXR-VDR complex with the VDRE in the promoter region of the *IFNγ* gene (Kongsbak et al., 2013).

The 1,25(OH)2D_3-RXR-VDR complex can also compete with transcription factors preventing them from binding to their promoter and initiating transcription (Kongsbak et al., 2013). Secretion of IL-2 is suppressed by this complex by antagonising the binding of nuclear factor of activated T cells (NFAT) to the distal end of the IL-2 promoter sequence (Seshadri, 2011). Similarly, secretion of IL-12 is suppressed by the complex binding to IL-12s transcription factor, nuclear factor kappa B (NFκB) (Kongsbak et al., 2013, Prietl et al., 2013).

Vitamin D_3 mediated suppression of these cytokines inhibits proliferation of the Th1 arm. Vitamin D_3 further promotes the production of anti- inflammatory Th2 cytokines (IL-3, IL-4, IL-5, IL-10 and TGFβ) resulting in a more tolerogenic immune status (Prietl et al., 2013, Harinarayan, 2014). This results in a shift from the Th1 to the Th2 pathway (Kamen and Tangpricha, 2010). Th2 supports humoral immunity by stimulating IgM, IgG1 and IgE synthesis by B lymphocytes and active eosinophils (Tisch and McDevitt, 1996, Azar et al., 1999). Vitamin D_3 ultimately reduces the number of APCs by inhibiting differentiation and maturation of dendritic cells that stimulate T cells, thus supporting T cell tolerance and the Th2 pathway (Altieri et al., 2017). Th1 cells are implicated in the development of autoimmunity suggesting a protective role for vitamin D_3 in autoimmune diseases such as T1D (Kamen and Tangpricha, 2010).

VDR plays an important role in mediating the immune response, and polymorphisms in this gene have been associated with the development of T1D (Kamen and Tangpricha, 2010, Seshadri, 2011).

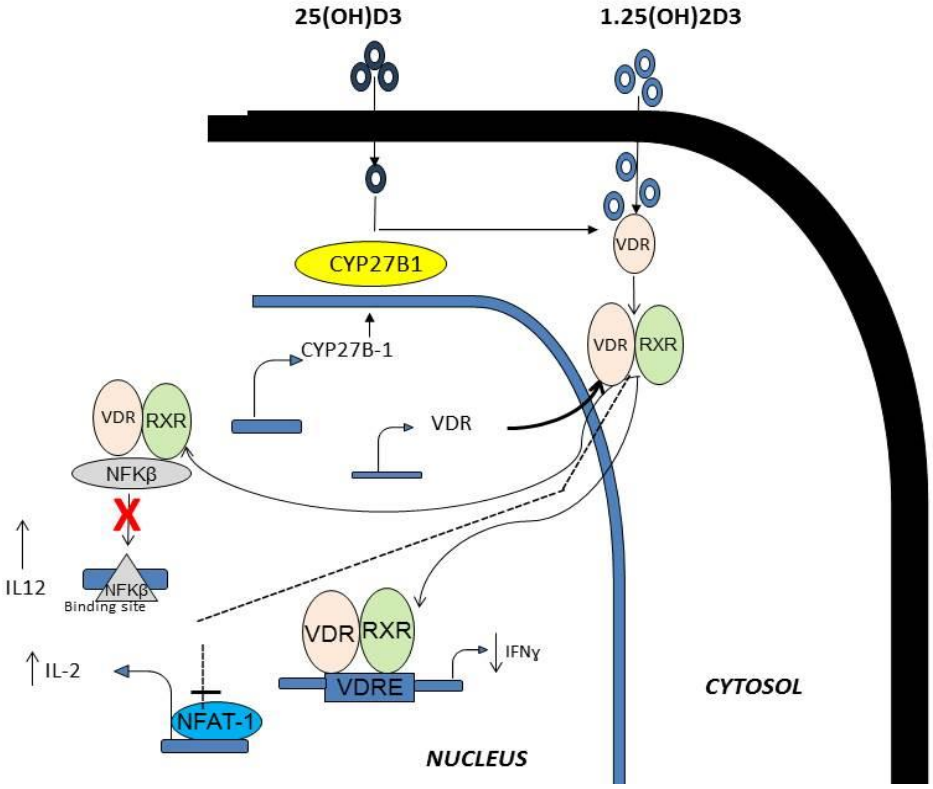


Figure 1.4: Proposed mechanism of vitamin D_3 signalling in T cells

The vitamin D-VDR-RXR complex binds to the VDRE down regulating *IFNɣ*. Similarly, the complex binds NFκB preventing it from binding to the NFκB binding site in the promoter region of IL-12 thus preventing IL-12 transcription. This complex further inhibits IL-2 expression by binding to the NFAT-1 binding site. The down regulation of *IL-2*, *IL-12* and *IFNɣ* shifts the T helper cells from the Th1 to the Th2 arm. Image adapted from Kongsbak et al. (2013).

1.3.5 The vitamin D receptor

The VDR belongs to the steroid receptor superfamily of transcription factors (Penna-Martinez and Badenhoop, 2017). It is composed of three domains, an N-terminal dual zinc finger DNA-binding domain, a C-terminal ligand-binding domain and an unstructured region that links the two functional domains (Penna-Martinez and Badenhoop, 2017, Pike and Meyer, 2010). The human *VDR* gene spans over 100 kilobases (kb) of genomic DNA and is located on chromosome 12q13.11. The *VDR* gene contains a 5' promoter region, eight protein-coding exons 2–9, six untranslated exons (1a–1f which are alternately spliced), introns and a 3′ untranslated region (UTR) (Mathieu and Badenhoop, 2005, Chakhtoura and Azar, 2013, Martin et al., 2010, Pike and Meyer, 2010).

Numerous single nucleotide polymorphisms (SNPs) have been identified within the *VDR* gene. Due to the role of the VDR in cytokine suppression, it is postulated that genetic variation in this gene may contribute to the development of T1D (Seshadri, 2011, Penna-Martinez and Badenhoop, 2017, Guo et al., 2006). There are four common SNPs of the *VDR* gene that have been investigated; namely, BsmI (rs1544410), FokI (rs2228570), ApaI (rs7975232) and TaqI (rs731236) (Mukhtar et al., 2017). These SNPs are commonly referred to by the name of the restriction enzymes used to genotype participants in PCR-RFLP analyses (Ginter and Simko, 2012). For the purpose of this dissertation, we will be referring to the SNPs using the enzyme names for identification.

1.3.6 Polymorphisms in the *VDR* gene

1.3.6.1 The BsmI polymorphism

The BsmI polymorphism is found near the 3' end of the *VDR* gene and is located in intron 8 and consists of a G to A substitution (Guo et al., 2006, Sahin et al., 2017, Penna-Martinez and Badenhoop, 2017). This SNP does not alter the structure, function or amount of the VDR protein produced (Raimondi et al., 2009, Penna-Martinez and Badenhoop, 2017). The BsmI SNP is in linkage disequilibrium with a poly(A) microsatellite repeat in the 3' untranslated region of the gene. This repeat influences VDR mRNA stability and VDR translational activity (Sahin et al., 2017, Guo et al., 2006, Uitterlinden et al., 2004b, Raimondi et al., 2009).

1.3.6.2 The FokI polymorphism

The FokI polymorphism in exon 2 causes a T to C substitution. FokI is bi-allelic and is also referred to as the start codon polymorphism (Uitterlinden et al., 2004a, Penna-Martinez and Badenhoop, 2017). This polymorphism eliminates the first start codon (ATG), thus transcription only begins at the alternative start codon six nucleotides downstream of the original start location. This results in a VDR protein that is three amino acids shorter than the wildtype protein (Penna-Martinez and Badenhoop, 2017, Sahin et al., 2017). The C allele, which generates the shorter variant (424 amino acids), is 1.5 to 2.5 fold more active than the longer variant (T allele; 427 amino acids) (Cooper et al., 2011, Ponsonby et al., 2008, Uitterlinden et al., 2004a). In addition to generating a more

active protein, the presence of the C allele results in an increased transcription rate of the *VDR* gene (Gross et al., 1998, Sahin et al., 2017).

1.3.6.3 The ApaI polymorphism

The ApaI SNP is found in intron 8, and is positioned at the 3' untranslated region of the gene. This SNP results in a C to A change and has no effect on the VDR protein structure. However, as with BsmI, the A allele is strongly linked to the presence of the poly(A) microsatellite repeat in the 3' untranslated region, thus regulating gene expression through modulation of mRNA (Penna-Martinez and Badenhoop, 2017, Kamel et al., 2014).

1.3.6.4 The TaqI polymorphism

The TaqI SNP is located in exon 9 of the gene and is a T to C (Penna-Martinez and Badenhoop, 2017, Kamel et al., 2014). This is a silent SNP as it does not alter the VDR protein structure. It is strongly linked to a poly(A) microsatellite repeat in the 3' untranslated region and like the ApaI and BsmI polymorphisms, modulation of mRNA regulates gene expression (Penna-Martinez and Badenhoop, 2017, Kamel et al., 2014, Sahin et al., 2017).

1.3.7 The association of *VDR* SNPs with the development of T1D

A T1D-VDR association was first made by McDermott and colleagues in 1997 (McDermott et al., 1997). Since then there have been numerous publications on the association of *VDR* SNPs and T1D including case control data sets, family studies and meta-analyses looking at different population groups (Penna-Martinez and Badenhoop, 2017) (Table 1.2). However, results have been inconsistent. The BsmI A allele has been shown to be the risk allele for the development of T1D in Asian and Chilean populations while no association was observed for this SNP in European and most Middle Eastern populations (Wang et al., 2014, Garcia et al., 2007). (Wang et al., 2014, Garcia et al., 2007, Ali et al., 2018). However, a new study in Saudi Arabia found the G allele to be the risk allele (Ali et al., 2018). The ApaI A allele has been shown to be the risk allele for the development of T1D (Kamel et al., 2014, Chang et al., 2000); however, not all studies have shown this association (Kamel et al., 2014, Chang et al., 2000, Mohammadnejad et

al., 2012, Turpeinen et al., 2003). Association studies for both FokI and TaqI have shown contradictory findings with both alleles identified as risk alleles depending on the population studied (Fichna et al., 2010, Bonakdaran et al., 2012, Mohammadnejad et al., 2012, Ali et al., 2018, Mukhtar et al., 2017).

Table 1.2: Association of *VDR* polymorphisms with T1D in different countries

SNP	Population	Risk allele	Reference
BsmI	Finnish	No association	Turpeinen et al. (2003)
	Taiwanese	A allele	Chang et al. (2000)
	East Asian	A allele	Wang et al. (2014)
	Chilean	A allele	Garcia et al. (2007)
	Iranian	No association	Mohammadnejad et al. (2012)
	Korean	A allele	Cheon et al. (2015)
	Saudi Arabia	G allele	Ali et al. (2018)
FokI	Finnish	No association	Turpeinen et al. (2003)
	West Asian	C allele	Wang et al. (2014)
	Dalmatian	T allele	Zemunik et al. (2005)
	Japanese	C allele	Ban et al. (2001)
	Iranian	No association	Mohammadnejad et al. (2012)
	Pakistan	C allele	Mukhtar et al. (2017)
	Saudi Arabia	C allele	Ali et al. (2018)
ApaI	Egyptian	A allele	Kamel et al. (2014)
	Taiwanese	A allele	Chang et al. (2000)
	Chilean	No association	Holick (2007)
	Iranian	No association	Mohammadnejad et al. (2012)
	Finnish	No association	Turpeinen et al. (2003)
TaqI	Iranian	C allele	Mohammadnejad et al. (2012)
	Iranian	No association	Bonakdaran et al. (2012)
	Egyptian	T allele	Kamel et al. (2014)
	Taiwanese	No association	Chang et al. (2000)
	Chilean	No association	Garcia et al. (2007)
	Korean	C allele	Cheon et al. (2015)

1.3.8 Polymorphisms in the vitamin D metabolic pathway and their association with T1D

Vitamin D metabolising enzymes belong to the cytochrome P450 superfamily. Cytochromes P450 are mono-oxygenases that catalyse many reactions such as metabolism and synthesis of lipids and steroids including vitamin D_3 (O'Neill et al., 2013, Penna-Martinez and Badenhoop, 2017). *CYP2R1* and *CYP27B1* are located on chromosomes 11p15.2 and 12q13.1-q13.3, respectively (Penna-Martinez and Badenhoop, 2017). Polymorphisms in both the *CYP2R1* (rs10741657; G>A) and *CYP27B1* (rs10877012; G>T) genes have been shown to be associated with T1D susceptibility (Rose et al., 2013). For the purpose of this dissertation, polymorphisms of the metabolising enzyme genes will be referred to by the name of the gene in which they are found.

1.3.8.1 The *CYP2R1* polymorphism

The *CYP2R1* gene is comprised of five exons and is approximately 15.5 kb in length (Hussein *et al*., 2012). A change in the gene sequence (G to A substitution) of *CYP2R1* in exon 2 (at amino acid 99) leads to a loss of 25-hydoxylase activity which results in low levels $1,25(OH)_2D_3$, thus increasing an individual's risk of developing T1D (Hussein et al., 2012, Ramos-Lopez et al., 2007a).

A study conducted on Egyptian children with T1D found that patients homozygous for the G allele of *CYP2R1* had a 2.6 fold increased risk of developing T1D. In addition, diabetic patients with the GG genotype had lower vitamin D levels compared to healthy children (Hussein et al., 2012). Studies on German T1D patients indicated that the G allele was associated with T1D susceptibility. In addition, patients with the GG or GA genotype had lower $25(OH)D_3$ levels compared to control participants (Ramos-Lopez et al., 2007a). Cooper and colleagues confirmed that the AA genotype of the *CYP2R1* gene conferred protection to TID in a British cohort (Cooper *et al*., 2011).

1.3.8.2 The *CYP27B1* polymorphism

The *CYP27B1* gene contains nine exons and eight introns and is over 4.8 kb in length. The rs10877012 polymorphism is found in the promoter region of *CYP27B1* (-1260 G>T)

(Penna-Martinez and Badenhoop, 2017, Lopez et al., 2004, Ramos-Lopez et al., 2007b). The presence of the *CYP27B1* G allele leads to a decrease in the amount of active 1α-hydroxylase resulting in decreased conversion of 25(OH)D to $1,25(OH)_2D_3$. Lower levels of $1,25(OH)_2D_3$ are believed to increase the risk of T1D development (Moran-Auth et al., 2013).

In a study conducted on Egyptian children with T1D, it was found that patients homozygous for the G allele of *CYP27B1* had a 3.7 fold increased risk of developing T1D (Ramos-Lopez et al., 2007b, Hussein et al., 2012). In a German study, T1D patients with the GG genotype had lower vitamin D_3 levels and showed decreased mRNA expression of the *CYP27B1* gene compared to healthy controls (Ramos-Lopez *et al*., 2007b). Similarly, British individuals carrying the G allele of the *CYP27B1* polymorphism had an increased risk of developing T1D (Bailey et al., 2007). However, in a Polish study no significant differences were found in *CYP27B1* allelic or genotypic frequencies between T1D patients and healthy controls (Fichna et al., 2010).

Despite numerous studies investigating the effect of vitamin D_3 concentrations, and *VDR* and vitamin D metabolising enzyme gene polymorphisms on T1D, there is no data in the black South African type 1 diabetic population. We therefore aimed to determine the role that vitamin D plays in the aetiology of T1D in black South African individuals.

1.4 Aim and Objectives

The main aim of this study was to determine whether polymorphisms in the genes regulating vitamin D function and metabolism play a role in the aetiology of T1D in the black South African population. Therefore, the specific objectives of the study were:

- To measure and compare serum vitamin D_3 concentrations in T1D patients (cases) and non-diabetic participants (controls)
- To screen participants for four polymorphisms in the *VDR* gene (BsmI [rs1544410], FokI [rs2228570], ApaI [rs7975232], TaqI [rs731236] and compare allelic and genotypic frequencies between cases and controls and relate these to serum vitamin D_3 levels
- To determine the prevalence of polymorphisms in the vitamin D metabolising enzyme genes (*CYP2R1* [rs10741657] and *CYP27B1* [rs10877012]) and compare allelic and genotypic frequencies between cases and controls and relate these to serum vitamin D_3 levels

- To determine GAD65, IA-2 and ZnT8 AAb positivity in cases and controls, compare AAb positivity across the genotypes of all polymorphisms studied and relate to serum vitamin D_3 levels

Chapter 2

2 Materials and Methods

The stock solutions and dilution preparations for all reagents used within this study can be found in Appendix A.

2.1 Study participants

Clinically diagnosed South African black T1D patients (cases; n=186) were recruited from diabetic clinics at Chris Hani Baragwanath Academic Hospital (CHBAH) and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Non-diabetic black individuals (controls; n=156) were recruited from the South African Blood Transfusion Services (SANBS) blood drives or from students and staff located at Wits Medical School and CMJAH. Control participants were included in the study if they had normal blood glucose levels (random glucose measurement ≤ 11.1mmol/L) and no family history of T1D, or any other autoimmune condition. Patients with clinical evidence of chronic pancreatitis, pregnancy related diabetes, T2D or those taking vitamin D supplements were excluded from the study.

To control for seasonal variation in vitamin D_3 levels, blood sampling of patients and controls were matched as closely as possible (i.e. recruited during the same seasonal time interval). Informed consent was obtained from all participants prior to enrolment into the study. Participants completed a questionnaire (Appendix B), anthropometric measurements were taken and the amount of sun exposure recorded.

The study protocol was approved by the Human Research Ethics Committee (M180334 and M150885) and by the SANBS Research Ethics Committee (clearance certificate number 2014/19). All ethics certificates can be found in Appendix C.

2.1.1 Calculation of sample size

The N number used in this study was calculated based on the ability to determine differences in allele frequencies between cases and controls for the polymorphisms with

the highest (62% for the A allele; rs7975232) and lowest (24% for the A allele; rs1544410) recorded disease-associated allele frequencies in African populations (Genomes Project et al., 2015). Thus, with an N of 150 participants in each group we will be able to detect an 11% difference in the A allele for rs7975232 between cases and controls and a 10% difference in the A allele for rs1544410 at $p < 0.05$.

2.1.2 Sample Collection

Blood samples (1 x 5 ml EDTA and 1 x SST serum tube (Becton Dickinson, New Jersey, USA)) were collected from all participants in the study. An additional Sodium Fluoride tube (Becton Dickinson, New Jersey, USA) was collected from control participants for glucose measurement to confirm normoglycaemia.

2.1.3 Sample Separation

Blood samples were spun down in the AllegraTM X 22R centrifuge (Beckman Coulter, California, USA) at 770 x *g* for 10 minutes to separate into the desired fractions. The top serum layer was removed from the SST tubes and aliquoted into 1.5 ml centrifuge tubes (Eppendorf, Hamburg, Germany). Similarly, the buffy coat was collected from EDTA tubes and aliquoted into 1.5 ml centrifuge tubes. All samples were stored in a Forma 900 Series freezer (Thermo Fisher Scientific, Waltham, MA, USA) at -80ºC until required.

2.2 DNA Extraction

Stored samples were removed from the -80ºC freezer and allowed to thaw at room temperature. DNA was extracted from 200 µl buffy coats using the Invisorb Spin Blood Mini DNA Extraction Kit (Stratatec Biomedical AG, Birkenfield, Germany) according to the manufacturer's instructions. All centrifugation steps were carried out using the Mikro 200 Hettich microcentrifuge (Hettich, Tuttlingen, Germany).

Briefly, 200 µl of buffy coat was added to a 1.5 ml microcentrifuge tube followed by 200 µl of lysis buffer. The tube was vortexed for 15 seconds and thereafter 20 µl of proteinase K added. Samples were vortexed and placed in a heating block for 10 minutes at 56°C. The tubes were removed from the heating block and 400 µl of binding buffer was added and the tube left to stand for one minute. The entire sample was then loaded onto the

spin filter. Tubes were centrifuged for two minutes at 11 000 x *g.* Collection tubes were discarded and the spin filters placed in a new receiver tube. Wash buffer 1 (500 µl) was added to each spin filter, centrifuged for one minute at 11 000 x *g*, and the receiver tube discarded. The spin filter was placed into a new receiver tube and 800 µl of wash buffer 2 added. Tubes were centrifuged for one minute at 11 000 x *g* and the flow-through discarded. Tubes were centrifuged for an additional four minutes at 11 000 x *g* to remove any excess ethanol. The spin filter was placed in a new collection tube and 150 µl elution buffer added. After a one minute incubation period at room temperature, the tubes were centrifuged for one minute at 11 000 x *g.* The DNA concentration was determined spectrophotometrically on the NanoDrop ND-1000 (NanoDrop Technologies, Delaware, USA). Samples were stored at -80ºC until required.

2.3 Detection of polymorphisms in the *VDR* and vitamin D metabolising enzyme genes

All study participants were genotyped for four SNPs (BsmI, FokI, ApaI and TaqI) in the *VDR* gene and an additional two SNPs in the *CYP27B1* and *CYP2R1* genes. The genotypes of the above-mentioned SNPs were determined by PCR based restriction fragment length polymorphism (PCR-RFLP). The literature refers to the *VDR* alleles based on the restriction enzyme site present e.g. The presence of the rs1544410 polymorphism is detected by restriction digest with BsmI and the alleles are therefore referred to as B (uncut) or b (cut). B corresponds to the A allele and b with the G allele. Similarly, rs2228570 (FokI) alleles are referred to as F (C allele) and f (T allele); rs7975232 (ApaI) alleles are referred to as A (A allele) and ‘a’ (C allele) and for rs731236 (TaqI) alleles are referred to as T (T allele) and t (C allele) (Lombard et al., 2006). BsmI b, FokI f, ApaI ‘a’ and TaqI T allele are found on the coding strand.

2.3.1 PCR amplification

PCR is the enzymatic amplification of a DNA fragment of interest using primers, deoxyribose nucleotide triphosphates (dNTPs), Taq polymerase and buffer. It involves an initial denaturation step followed by three steps i.e. denaturation, annealing and elongation which are repeated for 30 cycles. To ensure that all amplicons have fully extended, a final elongation step is incorporated. Within the PCR reaction, the polymerase buffer creates the optimal conditions in which the polymerase can function.

Forward and reverse primers bind to complimentary regions of the DNA sequence at specific positions to ensure amplification of the region of interest. dNTPs are the building blocks of DNA. Magnesium chloride ($MgCl_2$), present in the buffer, acts as a cofactor for the Taq polymerase (Roux, 2009). Taq polymerase functions to add nucleotides to a DNA template producing a double stranded PCR product. The DNA of interest serves as the template for the subsequent amplification reaction.

All PCR reactions were carried out in a final volume of 15 µl. A homemade master mix was used for all SNPs (Table 2.1) with the exception of BsmI and *CYP27B1* which were amplified using 2x Master Mix (New England Biolabs Inc., Massachusetts, USA) (Table 2.2). The primer sequences and resultant PCR product sizes are listed in Table 2.3.

Table 2.1: PCR reagents for the amplification of FokI, ApaI, TaqI and *CYP2R1* PCR products

Reagent	**Volume (1x)**
10x buffer (JMR Holdings, London, United Kingdom)	1.5 µl
dNTPs (8mM; Bioline, London, United Kingdom)	0.8 µl
$MgCl_2$ (25mM; Fermentas, Massachusetts, USA)	0.5 µl
Forward primer (10 pmol) (Inqaba Biotech, Pretoria, South Africa)	0.5 µl
Reverse primer (10 pmol) (Inqaba Biotech, Pretoria, South Africa)	0.5 µl
Supertherm Gold Taq polymerase (5U/µl) (JMR Holdings, London, United Kingdom)	0.15 µl
Distilled water	variable
DNA (approximately 40ng)	variable

Table 2.2: PCR reagents for the amplification of BsmI and *CYP27B1* PCR products

Reagent	**Volume (1x)**
2x MM NEB master mix (New England Biolabs Inc., Massachusetts, USA)	7.5 µl
Forward primer (10 pmol) (Inqaba Biotech, Pretoria, South Africa)	0.5 µl
Reverse primer (10 pmol) (Inqaba Biotech, Pretoria, South Africa)	0.5 µl
Distilled water	variable
DNA (approximately 40ng)	variable

Table 2.3: Primer sequences flanking polymorphisms within the *VDR* and vitamin D metabolising enzyme genes

Gene	SNP	Primer Sequence	Amplicon length
***VDR*[#]**	G>A BsmI	5′-CAACCAAGACTACAAGTACCGCGTCAGTGA-3′ 5′-AACCAGCGGGAAGAGGTCAAGGG-3′	822bp
***VDR*[#]**	T>C FokI	5′-AGCTGGCCCTGGCACTGACTCTTGCTCT-3′ 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′	273bp
***VDR*[#]**	C>A ApaI	5′-CAGAGCATGGACAGGGAGCAAG-3′ 5′-GCAACTCCTCATGGCTGAGGTCTCA-3′	745bp
***VDR*[#]**	T>C TaqI	5′-CAGAGCATGGACAGGGAGCAAG-3′ 5′-GCAACTCCTCATGGCTGAGGTCTCA-3′	745bp
***CYP2R1*[$]**	G>A	5′-GGGAAGAGCAATGACATGGA-3 5′-GCCCTGGAAGACTCATTTTG-3′	287bp
***CYP27B1*[%]**	G>T	5’-GTGTTCCCTAAGTGTTGTCTC-3′ 5′-GCTGACTCGGTCTCCTCTG-3′	187bp

bp = base pairs; [#] Primer sequences for the *VDR* SNPs were obtained from (El-Beshbishy et al., 2015, Mukhtar et al., 2017, Mohammadnejad et al., 2012); [$] Primer sequences for the *CYP2R1* SNP were obtained from (Hussein et al., 2012); [%] Primer sequences for the *CYP27B1* SNP were designed using Primer 3 software (Untergasser et al., 2012).

Approximately 40 ng of DNA was added to each reaction. A non-template control was included in each run to ensure no contamination occurred. PCR reactions were carried out in a C1000 or T1000 Thermal cycler (Bio-Rad Laboratories, California, USA). The optimal annealing temperature for each SNP was determined by gradient PCR where a single sample was run at different annealing temperatures until a single, specific band of the correct size could be identified. All samples were amplified using the optimised conditions outlined in Table 2.4.

Table 2.4: PCR conditions for the six SNPs investigated

Step	Temperature	Duration	SNP	
Initial denaturation	94ºC	11 min	All	
Amplification				x 1
-Denaturation	94ºC	30 sec	All	
-Annealing	59ºC	30 sec	*CYP27B1*	x 30
	60ºC	30 sec	BsmI, FokI	
	61ºC	30 sec	ApaI, TaqI, *CYP2R1*	
-Extension	68ºC	30 sec	BsmI, *CYP27B1*	
	72ºC	30 sec	FokI, ApaI, TaqI, *CYP2R1*	
Final extension	68ºC	5 min	BsmI, *CYP27B1*	x 1
	72ºC	5 min	FokI, ApaI, TaqI, *CYP2R1*	

*min= minute; sec = second

2.3.2 Agarose gel electrophoresis

To confirm successful amplification of the gene of interest, a 3 µl aliquot of each PCR product was mixed with 1 µl of loading buffer. Samples were run on a 1% agarose gel containing 1x Gel red (Biotium, California, USA) for visualisation of DNA under UV light. A DNA HyperLadder™ 100 (Bioline Reagents, London, UK) was included to verify the size of the PCR amplicon. The gel was run at 100V for 30 minutes and then visualised under UV light using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, California, USA).

2.3.3 Restriction endonuclease digestion of PCR products

PCR products (7 µl) were subjected to restriction endonuclease digestion to determine which allele/s were present in each participant. The digestion reaction was set up in a final volume of 20 µl according to Table 2.5.

Table 2.5: Reagents used for restriction endonuclease digestion of PCR products for the six SNPs investigated

SNP	Restriction enzyme* (final concentration)	Restriction enzyme volume	CutSmart Buffer (10x)	Incubation temperature (˚C)
BsmI	BsmI (10U)	1 µl	2 µl	37
FokI	FokI (5U)	1 µl	2 µl	37
ApaI	ApaI (10U)	1 µl	2 µl	37
TaqI	TaqI (10U)	1 µl	2 µl	65
CYP2R1	MnlI (5U)	0.5 µl	2 µl	37
CYP27B1	TfiI (10U)	0.5 µl	2 µl	37

* New England Biolabs Inc., Massachusetts, USA

Digestion reactions were incubated for two hours in a C1000/T100 thermal cycler. The fragments were resolved on a 2% agarose gel (run for one hour at 100V) and visualised on the ChemiDoc MP Imaging System. The resulting fragments were sized against a DNA HyperLadder™ 100 ladder and each individual scored for the presence or absence of the restriction enzyme cut site. The genotypes and their corresponding fragment sizes are listed in Table 2.6.

Table 2.6: PCR-RFLP fragment sizes and their corresponding genotypes for the six SNPs

SNP	Fragment size (bp)	Genotype
BsmI	651 and 171	GG
	822, 651 and 171	GA
	822	AA
FokI	206 and 67	TT
	273, 206 and 67	TC
	273	CC
ApaI	528 and 217	CC
	745, 528 and 217	CA
	745	AA
TaqI	494 and 251	TT
	494, 293, 251 and 201	TC
	293, 251 and 201	CC

Table 2.6: PCR-RFLP fragment sizes and their corresponding genotypes for the six SNPs continued

SNP	Fragment size (bp)	Genotype
CYP2R1	150,108 and 29	GG
	258, 150, 108 and 29	GA
	258 and 29	AA
CYP27B1	138 and 49	GG
	187, 138 and 49	GT
	187	TT

2.3.4 Sequencing of the PCR fragments

PCR products representing each genotype (determined through PCR-RFLP), for each SNP investigated, were sent for sequencing to Inqaba Biotech Southern Africa. The sequencing was performed to confirm that the correct region was amplified and the accuracy of the RFLP assay. Sequences were analysed using Sequencher software, version 4.7.

2.4 Measurement of vitamin D_3 levels

Vitamin D_3 levels were measured using the ClinRep High Performance Liquid Chromatography (HPLC) complete kit 25-OH-Vitamin D_2/D_3 (RECIPE, Munich, Germany) according to the manufacturer's instructions. Vitamin D_3 levels were assessed by measuring 25(OH)D_3 and 25(OH)D_2 as these analytes have a slower rate of clearance from the circulation than 1,25$(OH)_2D_3$.

Samples were extracted using a liquid-liquid extraction before being run on the instrument. Samples (200 µl of standards, controls or plasma (test sample)) were added to 250 µl of precipitation Reagent P. Ice cold Internal Standard (IS) (250 µl) was added to the mixture and vortexed for 30 seconds followed by centrifugation for five minutes at 10 000 x *g* (Mikro 200 Hettich microcentrifuge; Hettich, Tuttlingen, Germany). The supernatant (upper phase) was removed and sent to the routine laboratory for analysis on the Shimadzu Nextera Ultra Performance Liquid Chromatography (UPLC) instrument (Shimadzu Corporation, Kyoto Prefecture, Japan). Extracted samples were injected into an isocratic flow of mobile phase (RECIPE, Munich, Germany) and separation of

25(OH)D_3, internal standard and 25(OH)D_2 took place on a reverse phase column with UV detection at a wavelength of 264 nm.

Vitamin D_3 was quantitated by the Lab Solutions software (Shimadzu Corporation, Kyoto Prefecture, Japan) using peak height of the sample and comparing the response to that obtained on a standard curve. The IS is similar to the analyte in terms of behaviour during sample preparation and chromatography. Any losses during the sample preparation could therefore be determined by calculating the IS recovery (Table 2.7). Extrapolation to 100% recovery allowed the concentration of vitamin D_3 in the sample to be calculated. Results were rejected and the samples were rerun if the IS recovery was < 75% or > 125% of that of the highest standard.

Table 2.7: Calculations for determining recovery rate and vitamin D concentration

	Equation
Recovery rate	Area IS (sample)/Area IS (calibrator)
Vitamin D_3 concentration	Area (sample) x Concentration (calibrator)/Area (calibrator) x recovery rate

Vitamin D_3 status was based on the Chemical Pathology diagnostic laboratories reference levels for the test used (part of External Quality Assessment Scheme) i.e.
Levels > 72.5 nmol/L = sufficient
Levels between 52.5 – 72.5 nmol/L = insufficient
Levels < 50.0 nmol/L = deficient

2.5 Measurement of AAbs to GAD65, IA-2 and ZnT8

GAD65, IA-2 and ZnT8 AAb titres were measured by ELISA using KRONUS antibody kits (KRONUS Inc., Idaho, USA) and the plates read on the SYNERGY HT Micro plate reader (BIOTEK, Vermont, USA). The principle of the assay depends on the ability of the AAb to act divalently and form a bridge between the antigen (GAD65, IA-2 or ZnT8) coated on the ELISA plate wells and liquid phase biotin labelled GAD65, IA-2 or ZnT8. The AAb-biotin bound complex is then quantitated by the addition of streptavidin peroxidase and a colourogenic substrate (TMB). The reaction is stopped with the addition of sulphuric acid (H_2SO_4) and the absorbance read at 450nm. The absorbance of each well is directly proportional to the amount of antibody present.

2.5.1 Measurement of GAD65 AAbs

Serum samples and the ELISA kit components were equilibrated to room temperature (20 – 24°C) before starting the assay. All samples were set up in duplicate. Samples (25 µl; standards, controls, serum) were pipetted into the 96-well plate coated with the GAD65 antigen. The plate was covered and incubated at room temperature on the Thermostar orbital microplate plate shaker incubator (Labtek, Germany, UK) at 500 rpm for an hour. Samples were aspirated out of each well and the plate washed three times with 200 µl wash buffer using the ELx50 Plate washer (BIOTEK, Vermont, USA). Excess liquid was removed following the last wash by tapping the inverted plate on absorbent paper towel.

Reconstituted GAD65-biotin (100 µl) was pipetted into each well using a stepper pipette so that the antibodies could react divalently and form a bridge between the GAD65 coated on the ELISA plate and the liquid phase GAD-biotin. The plate was covered and incubated at 500 rpm for an hour. The washing procedure was repeated followed by the addition of 100 µl of reconstituted Streptavidin-peroxidase (SA-POD) into each well. The plate was incubated for 20 minutes at room temperature and the washing procedure repeated. This was followed by the addition of 100 µl peroxidase substrate (TMB) into each well. The plate was subsequently incubated in the dark for 20 minutes without shaking. The TMB enhances the reaction and quantifies the amount of antibody present with the development of a blue colour. The variation of the blue colour in every well is directly proportional to the amount of antibody present. The reaction was stopped with 100 µl stop solution (0.5M H_2SO_4) for accurate results and the plate shaken for approximately five seconds to ensure proper mixing. This caused the blue colour to turn yellow. The plate was placed on the SYNERGY HT Micro plate reader and the absorbance read at 450 nm within five minutes. The concentration of AAbs was determined based on the standard curve generated.

2.5.2 Measurement of IA-2 and ZnT8 AAbs

The methods used for the detection of both IA-2 and ZnT8 AAbs were identical, with the exception of the capture antibodies which were specific to IA-2 and ZnT8, respectively.

Serum samples were removed from the Forma 900 Series -80 ºC freezer (Thermo Fisher Scientific, Massachusetts, USA) and allowed to reach room temperature before starting

the assay. Samples were mixed by gentle inversion. Samples (50 µl; standards, controls and serum) were pipetted, in duplicate, into wells on the plate. Reaction enhancer (25 µl) was then added to each well, and the plate covered and samples mixed briefly. The plate was incubated overnight for 16 – 20 hours at 2 – 8°C.

Following the overnight incubation, all contents were aspirated from the wells and the wells washed three times with 200 µl wash buffer using the ELx50 Plate washer. Excess liquid was removed after the last wash by tapping the inverted plate on absorbent material. Reconstituted IA-2/ZnT8 biotin (100 µl) was added to each well and the plate incubated at 500 rpm for one hour on an ELISA plate shaker. Following incubation, the plate was washed as described above. 100 µl of a 1:20 dilution of the SA-POD reagent was pipetted into each well and incubated in the dark for 20 minutes at room temperature. A blue colour developed during this stage. Stop solution (0.5M H_2SO_4) was then added to each well and the plate shaken for approximately five seconds to ensure even mixing and to stop the reaction so that accurate results could be obtained. This caused the blue colour to turn yellow. The plate was then placed on the SYNERGY HT Micro plate reader (BIOTEK, Vermont, USA) and the absorbance read at 450 nm within five minutes. A calibration curve was plotted with the absorbance at 450 nm and the concentration of IA-2/ZnT8 AAbs determined.

2.6 Measurement of glucose concentrations

Glucose concentrations were measured on the Advia Chemistry System (Siemens Health Care Diagnostics Inc., New York, USA) in the National Health Laboratory Services (NHLS) Chemical Pathology Diagnostic Laboratory at the CMJAH. Sodium fluoride tubes (Becton Dickinson, New Jersey, USA) were centrifuged for 10 minutes at 770 x *g* (Beckman Coulter, California, USA) and the plasma sent to the routine laboratory for analysis.

The Hexokinase_3 method was used for the measurement of glucose concentrations. This is an enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase enzymes. The ADVIA Chemistry Glucose Hexokinase_3 method is a two component reaction. Sample was added to Reagent 1, which contained the buffer, adenosine triphosphate (ATP), and nicotinamide adenosine dinucleotide (NAD). Absorbance readings of the sample in Reagent 1 were taken and used to correct for interfering

substances in the sample. Reagent 2 was added, which initiated the reaction. Glucose was phosphorylated by ATP in the presence of hexokinase. The glucose-6-phosphate that formed was oxidised in the presence of glucose-6-phosphate dehydrogenase causing the reduction of NAD to nicotinamide adenosine dinucleotide hydrogenase (NADH). The absorbance of NADH was measured as an endpoint reaction at 340/410 nm. The difference between the absorbance in Reagent 1 and Reagent 2 is proportional to the glucose concentration.

2.7 Calculation of AAb positivity by hexiles based on duration of disease

T1D patients were divided into six groups (hexiles) based on disease duration (Appendix G).

Chapter 3

3 Results

This study aimed to explore whether vitamin D_3 levels and/or polymorphisms in genes involved in vitamin D_3 metabolism (*CYP2R1* and *CYP27B1*) and the receptor responsible for downstream signalling events (VDR) are associated with the development of T1D in the South African black population.

3.1 Characteristics of the study population

A total of 338 black South African participants were recruited for the study consisting of 182 T1D patients and 156 controls. However, three control participants were excluded from the study due to a high random fasting glucose level (≥ 11.1 mmol/L) suggesting that these participants were undiagnosed diabetics (American Diabetes Association, 2012). Thus, the final number of control participants was 153.

Participants recruited for the study ranged in age from 10 to 56 years with a median age of 27 [22; 35] years. There was an equal distribution of males and females in the study cohort (49.3% males, $p = 0.817$). Vitamin D_3 concentrations ranged from 21.31 to 159.61 nmol/L. Only 26.4% of participants had sufficient levels (> 72.5 nmol/L) of vitamin D_3, 38.1% had insufficient levels (52.5 - 72.5 nmol/L) and 35.4% of the cohort had deficient levels (< 52.5 nmol/L) of vitamin D_3.

Participants recruited during winter months had significantly lower vitamin D_3 levels when compared to participants recruited throughout the remainder of the year ($p < 0.001$); Figure 3.1). Vitamin D_3 deficiency varied according to season of sampling with 61.3% of those sampled in winter being deficient compared to 41.1% of those sampled in spring, 28.8% of those sampled in autumn and 25.5% in the summer.

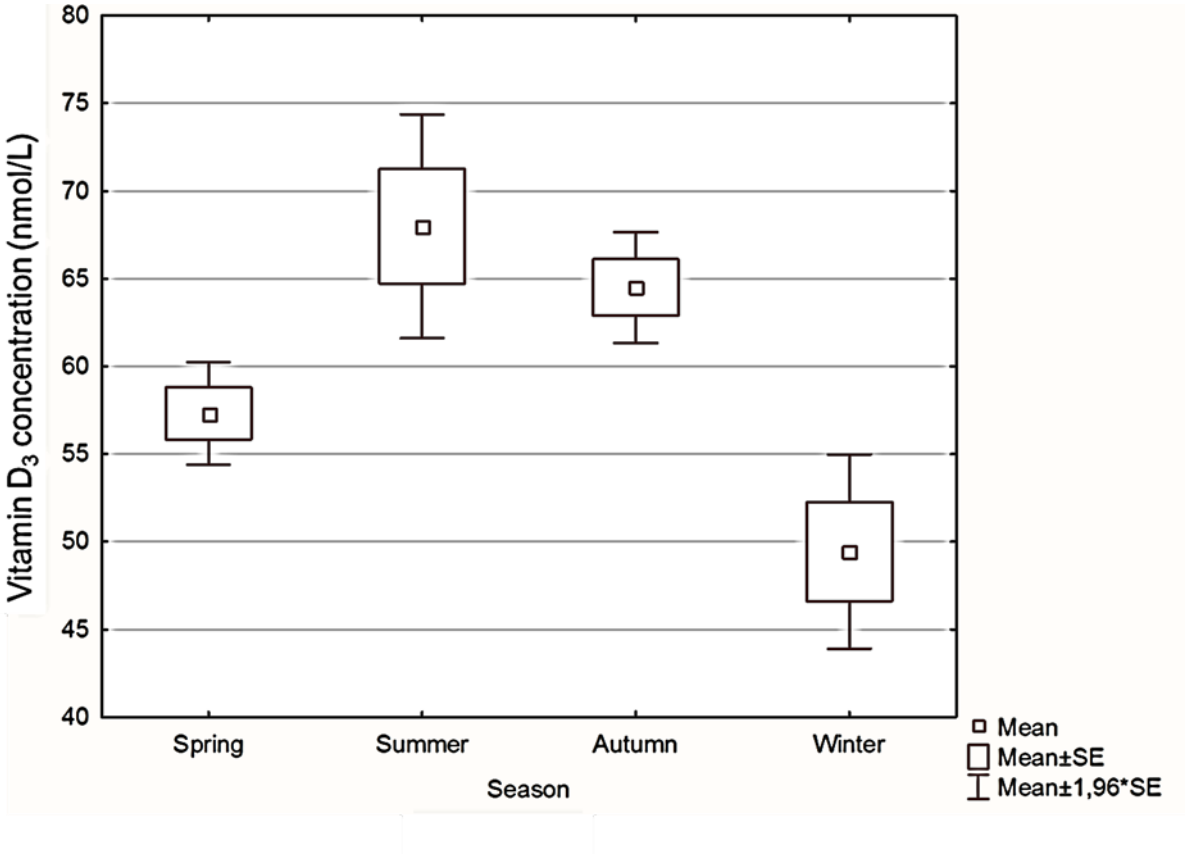


Figure 3.1: Categorical box and whisker plot showing vitamin D_3 concentrations (nmol/L) in our cohort according to the season of sampling

3.2 Clinical and phenotypic characteristics of T1D patients and control participants

The clinical and phenotypic characteristics of T1D patients and control participants are summarised in Table 3.1. Patients and controls were matched for age ($p = 0.487$). Within our cohort there were significantly more males in the T1D population compared to the control group ($p = 0.040$). Glucose levels were significantly higher in the patient group than the control group ($p < 0.001$). The majority of patients (85%) showed poor glycaemic control within the three months prior to sampling. Poor glycaemic control was defined as an HbA1c > 7 % (Webb et al., 2015). T1D patients had a significantly lower BMI ($p < 0.001$) and higher WHR than control participants ($p < 0.001$). No significant differences in serum vitamin D_3 levels were observed between patients and controls ($p = 0.110$). A significantly greater percentage of patients were GAD65 and ZnT8 AAb positive compared to control participants ($p < 0.001$ and $p = 0.016$, respectively). No significant difference in IA-2 AAb positivity was observed. The mean age at diagnosis in T1D patients

was 20.7 ± 8.4 years and the median duration of the disease at time of sampling was 7 [2; 11] years. Only 12.2 % of patients were recruited within a year of T1D diagnosis.

Table 3.1: Clinical and phenotypic characteristics of T1D patients and control participants

Variables	**T1D patients n = 182**	**Controls n = 153**	**p value**
Age (years)	27 [22; 35]	26 [22; 35]	0.487
Age at diagnosis (years)	20.7 ± 8.4	-	-
Duration of disease (years)	7 [2; 11]	-	-
Gender (% male)	54.4	43.1	**0.040***
Glucose (mmol/L)	9.2 [5.7; 13.2]	5.6 [4.7; 7.6]	**<0.001***
[a]HbA1c (%)	10.6 ± 3.4	-	-
BMI (kg/m^2)	23.7 [21.1; 28.0]	26.5 [23.4; 32.5]	**<0.001***
WHR	0.82 ± 0.08	0.78 ± 0.08	**<0.001***
Vitamin D_3 (nmol/L)	62.7 ± 20.7	59.4 ± 17.0	0.110
GAD65 AAb positivity (%)	51.0	4.3	**<0.001***
IA-2 AAb positivity (%)	12.9	6.8	0.103
ZnT8 AAb positivity (%)	17.6	4.0	**0.016***

Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, a HbA1c = glycated haemoglobin, WHR = waist hip ratio, * indicates significant values p<0.05

3.3 Age at Diagnosis

The age at diagnosis of disease within our cohort ranged from 0 to > 39 years with the majority (42.8 %) of individuals being diagnosed between the ages of 15 and 24 years (Figure 3.2). Only 24.4 % of patients were diagnosed before 15 years of age.

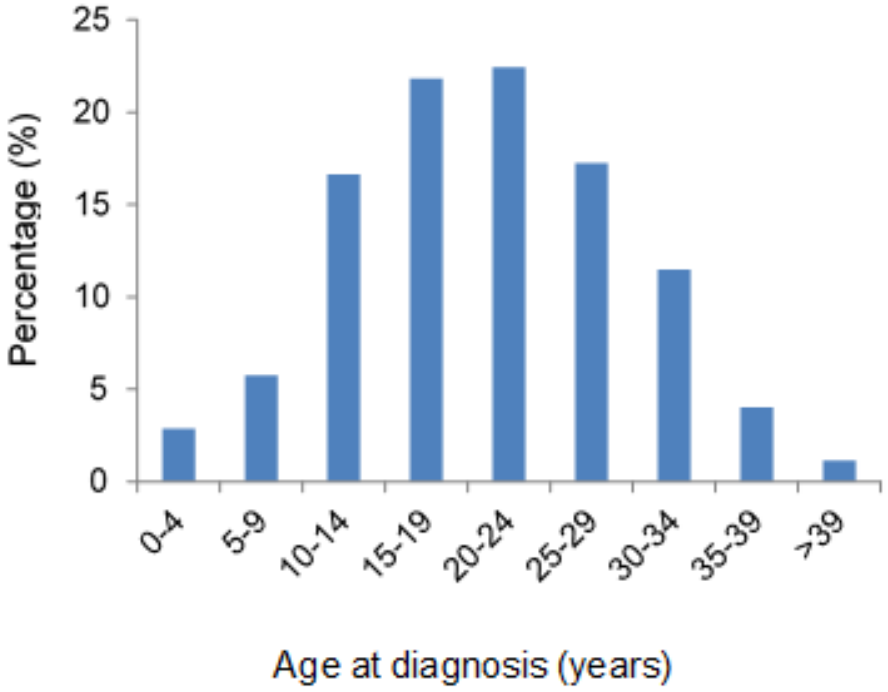


Figure 3.2: Graphical representation of the percentage of black South African T1D patients by age at diagnosis

3.4 Detection of polymorphisms in the *VDR* and vitamin D metabolising enzyme genes in all study participants

The genotypes for the six SNPs investigated (BsmI, FokI, ApaI, TaqI, *CYP2R1* and *CYP27B1*) were determined by PCR–RFLP. The restriction fragment patterns obtained following restriction enzyme digestion with BsmI, FokI, ApaI, TaqI, MnlI (*CYP2R1*) and TfiI (*CYP27B1*) are illustrated in Figure 3.3 and 3.4, respectively. Gel images for the PCR products obtained for the six SNPs studied can be seen in Appendix D.

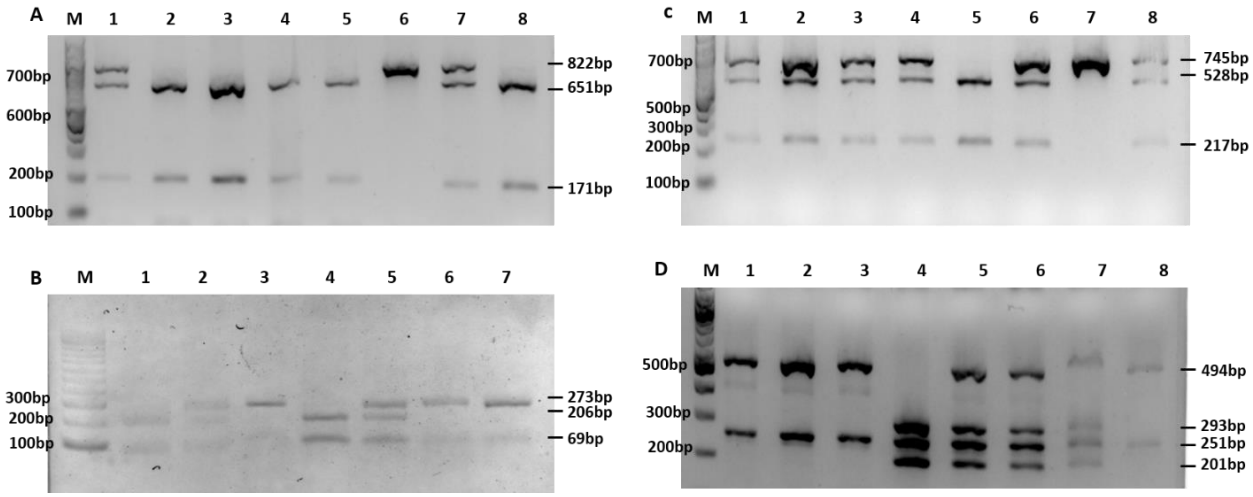


Figure 3.3: Restriction digestion profile of the four *VDR* polymorphisms run on a 2 % agarose gel.

(A) BsmI digestion profile: Lane M: 100bp DNA ladder; Lane (1, 4 - 7): homozygous GG genotype - fragments of 651 and 171 bp; Lane (2, 8): heterozygous GA genotype - fragments of 822, 651 and 171 bp; Lane 3: homozygous AA genotype - fragment of 822 bp.
(B) FokI digestion profile: Lane M: 100bp DNA ladder. Lane (4, 7): homozygous TT genotype - fragments of 206 and 67 bp; Lane (1, 2, 5): CC homozygous FokI genotype – fragments of 273 bp; Lane (3 and 6): heterozygous TC genotype - fragments of 273, 206 and 67 bp.
(C) ApaI digestion profile: Lane M: 100bp DNA ladder; Lane (4): homozygous CC genotype - fragments of 528 and 217 bp; Lane (1, 3, 5-8): heterozygous CA genotype - fragments of 745, 527 and 217 bp; Lane 2: homozygous AA genotype - fragment of 745 bp.
(D) TaqI digestion profile: Lane M: 100bp DNA ladder; Lane (1, 6-8): homozygous TT genotype - fragment of 494 and 251 bp; Lane (5): homozygous CC genotype - fragments of 293; 251 and 201 bp; Lane (3, 4): heterozygous TC genotype with fragments of 494, 293, 251 and 201 bp.

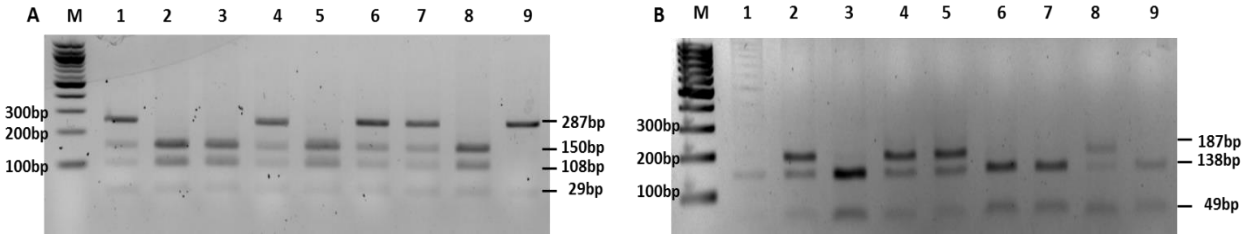


Figure 3.4: Restriction digestion profile of the two metabolising enzyme gene polymorphisms run on a 2 % agarose gel.

(A) MnlI (*CYP2R1*) digestion profile: Lane M: 100bp DNA ladder; Lane (1, 4, 6, 7): heterozygous AG genotype - fragments of 258, 150, 108 and 29 bp; Lane (2, 3, 5, 8): homozygous GG genotype - fragments of 254 and 33 bp; Lane 9: Homozygous AA genotype - fragment of 287 bp.
(B) TfiI (*CYP27B1*) digestion profile: Lane M: 100bp DNA ladder; Lane (1, 3, 6, 7, 9): GG genotype of *CYP27B1* – fragments of 138 and 49 bp; Lane (2, 4, 5 8): heterozygous GT genotype - fragments of 187, 137 and 49 bp.

3.4.1 Sequencing

Three samples, representing the three different genotypes, for each SNP were selected for sequencing to confirm the PCR - RFLP results. The results of the sequencing for the ApaI SNP are shown in Figure 3.5.

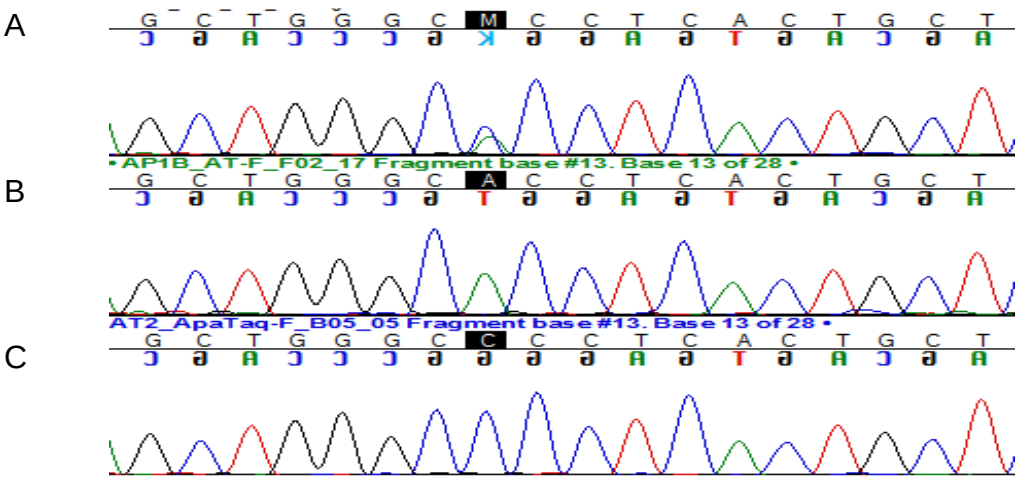


Figure 3.5: Chromatograms obtained from the amplification of the *VDR* gene containing the ApaI polymorphism

A: chromatogram illustrating a heterozygous CA individual for the ApaI SNP

B: chromatogram illustrating a homozygous AA individual for the ApaI SNP

C: chromatogram illustrating a homozygous CC individual for the ApaI SNP

3.4.2 Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in T1D patients and control participants

The T1D and control groups were in Hardy Weinberg equilibrium for all four variants of the *VDR* gene as well as the two variants of the metabolising enzyme genes (Appendix E). Table 3.2 summarises the genotypic and allelic frequencies obtained for the six polymorphisms studied in the T1D patients and control group. There were no significant differences seen in genotypic or allelic frequencies between T1D patients and controls in the four *VDR* and two metabolising enzyme gene SNPs investigated. Thus, these SNPs are not associated with the development of T1D in the black South African population.

Table 3.2: Genotypic and allelic frequencies of *VDR* and vitamin D metabolising enzyme gene polymorphisms in T1D patients and controls

SNP	T1D patients	Controls	p value
	Frequency (n)	Frequency (n)	
BsmI			
Genotype model			
GG	0.67 (120)	0.61 (85)	0.474
GA	0.28 (49)	0.34 (47)	
AA	0.05 (9)	0.05 (7)	
Allele model			
G	0.81 (289)	0.79 (217)	0.331
A	0.19 (67)	0.21 (61)	
FokI			
Genotype model			
TT	0.02 (4)	0.05 (8)	0.247
CT	0.29 (53)	0.31 (47)	
CC	0.69 (126)	0.63 (95)	
Allele model			
T	0.17 (61)	0.21 (63)	0.153
C	0.83 (305)	0.79 (237)	
ApaI			
Genotype model			
CC	0.13 (24)	0.09 (13)	0.389
CA	0.43 (78)	0.43 (64)	
AA	0.44 (79)	0.48 (72)	
Allele model			
C	0.35 (126)	0.30 (90)	0.210
A	0.65 (236)	0.69 (208)	
TaqI			
Genotype model			
TT	0.69 (123)	0.60 (89)	0.189
TC	0.27 (48)	0.36 (54)	
CC	0.04 (7)	0.04 (6)	
Allele model			
T	0.83 (294)	0.78 (232)	0.129
C	0.17 (62)	0.22 (66)	

Table 3.2: Genotypic and allelic frequencies of *VDR* and vitamin D metabolising enzyme gene polymorphisms in T1D patients and controls continued

SNP	T1D patients	Controls	p value
	Frequency (n)	Frequency (n)	
CYP2R1			
Genotype model			
GG	0.63 (113)	0.66 (100)	0.106
GA	0.32 (58)	0.33 (50)	
AA	0.04 (8)	0.01 (1)	
Allele model			
G	0.79 (284)	0.83 (250)	0.261
A	0.21 (74)	0.17 (52)	
CYP27B1			
Genotype model			
GG	0.81 (147)	0.85 (129)	0.261
GT	0.18 (35)	0.13 (20)	
TT	0.0 (0)	0.01 (2)	
Allele model			
G	0.90 (329)	0.92 (278)	0.451
T	0.10 (35)	0.08 (24)	

3.4.3 Associations of the *VDR* and vitamin D metabolising enzyme gene polymorphisms with vitamin D_3 levels in black South African participants

The FokI and TaqI polymorphisms within the *VDR* gene showed an association with vitamin D_3 levels (Table 3.3). The vitamin D_3 levels for the CC genotype of FokI were significantly lower than those for participants with the TC and TT genotypes (59.28 ± 18.18 vs. 65.12 ± 20.66 nmol/L; p = 0.009). Similarly, the vitamin D_3 levels for the CC/TC combined genotypes of TaqI were significantly lower than those for participants with the TT genotype (58.37 ± 18.53 vs. 62.83 ± 19.54 nmol/L; p = 0.047). The remaining *VDR* polymorphisms did not show an association with vitamin D_3 levels. Similarly, no associations were seen with the metabolising enzyme gene polymorphisms and vitamin D_3 levels (Table 3.4).

Table 3.3: Associations of the *VDR* polymorphisms with vitamin D_3 levels in black SA participants

SNP	Genotype (n)	Vitamin D_3 (nmol/L)	p value
BsmI[#]	GG (205) AG + AA (112)	61.88 ± 19.21 60.55 ± 19.46	0.560
FokI[#]	CC (221) TC + TT (112)	59.28 ± 18.18 65.12 ± 20.66	**0.009***
ApaI	CC (37) CA (142) AA (151)	63.16 ± 16.96 61.76 ± 18.57 60.43 ± 20.28	0.691
TaqI[#]	TT (212) TC + CC (115)	62.83 ± 19.54 58.37 ± 18.53	**0.047***

Results are presented as mean ± standard deviation for non-skewed data, # genotypes were combined due to the small n number for the homozygous A, T and C genotypes for the BsmI, FokI and TaqI SNPs, respectively, * indicates significant values

Table 3.4: Associations of the metabolising enzyme gene polymorphisms with vitamin D_3 levels in black SA participants

SNP	Genotype	Vitamin D_3 (nmol/L)	p value
CYP2R1[#]	GG (GG) GA + AA (117)	61.32 ± 19.80 60.72 ± 18.14	0.786
CYP27B1[#]	GG (276) GT + TT (55)	61.59 ± 19.48 59.89 ± 17.56	0.544

Results are presented as mean ± standard deviation for non-skewed data, # genotypes were combined due to the small n number for the homozygous A and T genotypes for the *CYP2R1* and *CYP27B1* SNPs, respectively, * indicates significant values

Although no difference in genotypic or allelic frequencies were observed between T1D patients and control participants (Table 3.2) we subsequently investigated whether any associations existed between the genotypic frequencies obtained for the six polymorphisms and clinicopathological variables in the T1D patients.

3.4.4 *VDR* and vitamin D metabolising enzyme genotypes and clinicopathological variables in T1D patients

The *VDR*, *CYP2R1* and the *CYP27B1* metabolising enzyme gene polymorphisms and their association with different clinicopathological variables are summarised in Table 3.5 and 3.6, respectively. The *VDR* genotypes showed no statistically significant difference with any of the clinicopathological variables studied. Similarly, there were no significant associations observed with the *CYP2R1* and the *CYP27B1* metabolising enzyme genotypes and any of the variables investigated.

However, patients with the *CYP2R1* GG genotype showed a tendency towards lower ZnT8 AAb positivity when compared to patients with either AA or AC genotypes (13.3 % vs. 24.2 % positivity; p = 0.061). Similarly, patients with the *CYP27B1* GG genotype showed a tendency towards lower ZnT8 AAb positivity when compared to patients with either the TT or TC genotypes (15.0 % vs. 28.6 % positivity; p = 0.057).

Table 3.5: Associations of the *VDR* polymorphisms with clinicopathological variables in T1D patients

Variable	BsmI genotype[#]			FokI genotype[#]		
	GG	AA+ GA	p value	CC	CT + TT	p value
Age at diagnosis (years)	21.25 ± 7.65	19.26 ± 9.18	0.111	21.86 ± 8.41	20.19 ± 8.30	0.215
Male (%)	53.3	58.6	0.506	50.9	56.0	0.520
Glucose (mmol/L)	8.3 [2.42; 32.17]	10.2 [2.95; 28.62]	0.240	9.2 [2.48; 32.22]	9.25 [2.42; 22.01]	0.587
HbA1c (%)	10.61 ± 3.52	10.44 ± 3.19	0.772	11.03 ± 3.67	10.42 ± 3.29	0.275
BMI (kg/m^2)	23.18 [16.27; 45.68]	23.85 [14.99; 44.40]	0.869	23.73 [14.99; 45.61]	23.14 [16.34; 40.08]	0.387
WHR	0.82 ± 0.07	0.82 ± 0.08	0.829	0.82 ± 0.07	0.82 ± 0.08	0.961
Vitamin D_3 (nmol/L)	63.57 ± 20.81	61.60 ± 20.99	0.556	68.17 ± 22.96	60.27 ± 19.23	**0.017***
GAD65 positivity (%)	52.0	49.1	0.730	49.1	55.3	0.474
IA-2 positivity (%)	16.0	8.5	0.162	11.1	17.0	0.313
ZnT8 positivity (%)	20.8	12.0	0.154	15.2	22.8	0.211

Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, # genotypes were combined due to the small n number for the homozygous A, T and C genotypes for the BsmI, FokI and TaqI SNPs, respectively,* indicates significant value

Table 3.5: Associations of the *VDR* polymorphisms with clinicopathological variables in T1D patients continued

Variable	ApaI genotype				TaqI genotype#		
	CC	CA	AA	p value	TT	TC + CC	p value
Age at diagnosis (years)	21.17 ± 10.01	20.46 ± 8.02	0.81 ± 8.28	0.928	20.89 ± 8.24	20.35 ± 8.76	0.689
Male (%)	45.8	56.4	54.4	0.660	52.8	56.4	0.664
Glucose (mmol/L)	10.40 [5.70; 16.80]	8.05 [5.25; 13.00]	9.75 [6.10; 13.30]	0.215	8.2 [2.78; 23.43]	9.85 [2.36; 32.17]	0.248
HbA1c (%)	10.77 ± 3.52	10.68 ± 3.69	10.56 ± 3.13	0.962	10.39 ± 3.34	11.27 ± 3.56	0.128
BMI (kg/m^2)	23.73 [20.99; 25.51]	22.70 [21.30; 28.72]	23.96 [21.06; 27.96]	0.856	23.53 [16.07; 45.75]	23.49 [15.06; 44.40]	0.729
WHR	0.81 ± 0.07	0.82 ± 0.06	0.82 ± 0.09	0.744	0.82 ± 0.08	0.82 ± 0.07	0.625
Vitamin D$_3$ (nmol/L)	61.83 ± 19.44	65.06 ± 20.57	60.97 ± 21.35	0.454	63.53 ± 21.36	60.65 ± 19.72	0.396
GAD65 positivity (%)	54.5	50.7	50.8	0.947	49.5	54.2	0.594
IA-2 positivity (%)	22.7	7.2	14.3	0.131	11.7	14.6	0.613
ZnT8 positivity (%)	29.2	11.5	19.0	0.113	17.9	14.5	0.582

Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, # genotypes were combined due to the small n number for the homozygous A, T and C genotypes for the BsmI, FokI and TaqI SNPs, respectively,* indicates significant value

Table 3.6: Associations of the metabolising enzyme gene polymorphisms with clinicopathological variables in T1D patients

Variable	*CYP2R1*			*CYP27B1*		
	GG	**AA + GA**	**p value**	**GG**	**TT + GT**	**p value**
Age at diagnosis (years)	20.42 ± 8.18	21.22 ± 8.81	0.543	20.83 ± 8.60	20.18 ± 7.25	0.683
Male (%)	59.3	47.0	0.110	55.1	51.4	0.695
Glucose (mmol/L)	9.45 [5.4; 13.2]	8.3 [5.7; 13.0]	0.947	9.25 [5.8; 13.3]	8.20 [5.4; 14.9]	0.452
HbA1c (%)	10.71 ± 3.54	10.42 ± 3.17	0.591	10.56 ± 3.43	10.89 ± 3.40	0.612
BMI (kg/m^2)	23.13 [20.43; 28.02]	23.83 [21.73; 27.44]	0.702	23.24 [20.96; 27.64]	23.83 [22.22; 30.54]	0.083
WHR	0.82 ± 0.08	0.81 ± 0.07	0.531	0.82 ± 0.08	0.81 ± 0.07	0.635
Vitamin D$_3$ (nmol/L)	64.08 ± 21.78	60.40 ± 18.89	0.255	63.88 ± 21.75	57.98 ± 15.14	0.130
GAD65 positivity (%)	47.9	57.1	0.272	51.2	50.0	0.910
IA-2 positivity (%)	11.5	16.1	0.417	11.8	17.9	0.388
ZnT8 positivity (%)	13.3	24.2	0.061	15.0	28.6	0.057

Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, [#] genotypes were combined due to the small n number for the homozygous A and T genotypes for the *CYP2R1* and *CYP27B1* SNPs, respectively

3.5 Association of *VDR* risk alleles with vitamin D_3 levels in the total cohort

The A, C, A and C alleles for the BsmI, FokI, ApaI and TaqI SNPs, respectively are the most common alleles that have been shown to be associated with T1D in the literature (see Table 1.2). Each individual can, therefore, have a maximum of eight *VDR* risk alleles and a minimum of zero. When participants were grouped according to the number of these T1D *VDR* risk alleles, vitamin D_3 levels were found to decrease with an increasing number of risk alleles ($p = 0.006$). Vitamin D_3 levels decreased from 66.53 ± 23.00 nmol/L (1 - 2 risk alleles) to 47.84 ± 13.80 nmol/L (7 – 8 risk alleles; Table 3.7).

Table 3.7: Associations of the *VDR* gene polymorphisms with clinicopathological variables in the total cohort

***VDR* gene risk alleles**[#]	**n**	**Vitamin D_3 levels (nmol/L)**	**p value**
1 - 2	65	66.53 ± 23.00	**0.006***
3 - 4	156	60.20 ± 16.64	
5 - 6	73	62.01 ± 20.83	
7 - 8	14	47.81 ± 13.80	

Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, # genotypes were combined due to the small n number for the homozygous A and T genotypes for the *CYP2R1* and *CYP27B1* SNPs, respectively

3.6 Association of Vitamin D_3 levels with increasing number of metabolising enzyme gene risk alleles in the total study cohort

The most common risk alleles for *CYP2R1* and *CYP27B1* have been shown to be the G alleles (Cooper et al., 2011, Hussein et al., 2012, Ramos-Lopez et al., 2007b, Ramos-Lopez et al., 2007a, Moran-Auth et al., 2013, Fichna et al., 2010). Each individual can have a maximum of four metabolising enzyme gene risk alleles and a minimum of zero. No association was seen between the number of risk alleles and vitamin D_3 levels ($p = 0.122$; Table 3.8).

Table 3.8: Association of vitamin D_3 levels and increasing number of metabolising enzyme gene risk alleles in the total cohort

Metabolising enzyme gene risk alleles[#]	**n**	**Vitamin D_3 levels (nmol/L)**	**p value**
0-1	4	67.26 ± 15.94	0.122
2	25	62.93 ± 17.94	
3	119	58.63 ± 18.25	
4	178	62.52 ± 19.94	

* The *VDR* risk alleles were classified as A for the BsmI SNP, C for FokI SNP, A for ApaI and C for TaqI. Results are presented as mean ± standard deviation for non-skewed data, * indicates significant values

3.7 Number of *VDR* and vitamin D metabolising enzyme gene risk alleles and their association with T1D patients

We then looked to see if there was any association between the number of *VDR* and vitamin D metabolising enzyme gene risk alleles in the T1D patients with age at diagnosis, glucose levels, vitamin D_3 concentrations and percentage HbA1c (Table 3.9 and Table 3.10, respectively). None of the variables were found to be associated with an increased number of *VDR* risk alleles. Similarly, no associations with any variables were seen with an increasing number of metabolising enzyme risk alleles.

Table 3.9: Associations between the number of *VDR* gene risk alleles and T1D related variables

Variable	Number of *VDR* risk alleles[#]				p value
	1-2 (n)	3-4 (n)	5-6 (n)	7-8 (n)	
Age at diagnosis (years)	20.33 ± 7.76 (40)	20.93 ± 7.90 (83)	20.28 ± 9.65 (40)	19.89 ± 8.59 (9)	0.961
Glucose (mmol/L)	10.15 [5.40;14.10] (38)	8.20 [5.30;13.20] (77)	9.85 [6.60;13.20] (38)	12.05 [5.10;12.90] (6)	0.867
HbA1c (%)	10.84 ± 3.37 (38)	10.50 ± 3.58 (80)	10.48 ± 3.37 (36)	11.30 ± 1.52 (5)	0.920
Vitamin D_3 (nmol/L)	68.15 ± 26.62 (41)	61.86 ± 16.38 (83)	63.29 ± 27.68 (40)	47.30 ± 14.87 (9)	0.051

[#] The *VDR* risk alleles were classified as A for the BsmI SNP, C for FokI SNP, A for ApaI and C for TaqI. Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, * indicates significant values

Table 3.10: Associations between the number of vitamin D metabolising enzyme gene risk alleles and T1D related variables

Variable	Number of metabolising gene risk alleles[#]				p value
	0-1 (n)	2 (n)	3 (n)	4 (n)	
Age at diagnosis (years)	19.00 ± 2.65 (3)	18.94 ± 7.85 (16)	22.02 ± 8.77 (66)	20.01 ± 8.25 (92)	0.383
Glucose (mmol/L)	10.80 [9.70;19.20] (3)	8.20 [5.70;15.00] (14)	8.20 [5.40;12.80] (61)	10.00 [5.80;14.50] (86)	0.431
HbA1c (%)	8.93 ± 1.88 (3)	10.20 ± 3.40 (15)	10.78 ± 3.34 (60)	10.56 ± 3.50 (86)	0.777
Vitamin D_3 (nmol/L)	63.49 ± 17.23 (3)	60.83 ± 15.91 (16)	58.27 ± 19.25 (67)	66.21 ± 22.36 (92)	0.122

[#] The metabolising enzyme risk alleles were classified as G for the *CYP2R1* SNP and for the *CYP27B1* SNP. Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, * indicates significant values

3.8 Multivariable linear regression model to determine which variables contribute to vitamin D_3 levels in the total cohort

Upon multiple regression analysis, sampling in spring and winter were found to be the strongest factors affecting vitamin D_3 concentrations in the total cohort (Table 3.11). Winter (beta = -18.20; $p < 0.001$) and spring (beta = -7. 66; $p = 0.001$) sampling were significant predictors of lower vitamin D_3 levels i.e. vitamin D_3 levels were 7.66 nmol/L lower in spring and 18.20 nmol/L lower in winter when compared to summer months.

Vitamin D_3 levels were 5.31 nmol/L higher in the male population in our cohort than the female cohort (beta = 5.31; $p = 0.009$).

Individuals with the FokI combined CT and TT genotype had 5.34 nmol/L higher vitamin D_3 levels than those with the CC genotype (beta = 5.34; $p = 0.013$). The C allele has been noted as the risk allele.

Variables included in the regression analysis were BMI, gender, season, skin exposure, time outdoors, genotype, diabetic status and age.

Table 3.11: Multivariable linear regression model to determine effects of different

Dependant variables	**Independent Variables**	**b Value**	**p value**	**(R) and (p) value for whole model**
Vitamin D_3 (n=334)	Spring	-7.66	**0.001***	0.372 (<0.001)
	Winter	-18.20	**<0.001***	
	Patient	4.94	**0.021***	
	Male	5.31	**0.009***	
	FokI CT/TT	5.34	**0.013***	
	TaqI TT	3.99	0.060	

* indicates significant values

3.9 GAD65 AAb positivity is predictive of the development of T1D

Logistic regression analysis showed that individuals who were shown to be GAD65 AAb positive were 23 fold more likely to develop T1D than GAD65 AAb negative individuals (confidence intervals 8.97 – 60.45; $p < 0.001$).

3.10 Association of GAD65, IA-2 and ZnT8 AAb positivity with clinicopathological variables in T1D patients

T1D patients positive for IA-2 AAbs were shown to have a significantly younger age at diagnosis than IA-2 AAb negative patients (16.40 ± 5.53 vs. 21.62 ± 8.56 years; $p = 0.009$) (Table 3.12). Similarly, ZnT8 AAb positivity was associated with a younger age at diagnosis (17.00 ± 6.49 vs. 21.48 ± 8.50 years; $p = 0.006$). The same trend was observed in GAD65 AAb positive patients however this did not reach significance (19.68 ± 8.70 vs. 22.28 ± 7.89 years; $p = 0.055$).

GAD65 AAb positivity was associated with a lower BMI (22.49 [20.96; 25.39] vs. 24.96 [21.72; 29.72]; $p = 0.021$). A similar association was seen for ZnT8 AAb positivity (BMI: 22.05 [19.39; 23.86] vs. 23.84 [21.38; 28.73]; $p = 0.003$). In addition, AAb positive patients were significantly younger than AAb negative patients (GAD65: $p < 0.001$; IA-2: $p < 0.001$; ZnT8: $p < 0.001$). ZnT8 AAb positivity was further associated with higher concentrations of glucose (10.85 [7.1; 16.1] vs. 8.4 [5.4; 13.2]; $p = 0.045$).

IA-2 negative patients had significantly lower vitamin D_3 levels than those with IA-2 AAbs ($p = 0.035$).

Table 3.12: Clinicopathological variables according to GAD65, IA-2 and ZnT8 AAb status in T1D patients

Variable	GAD65 AAb positivity			IA-2 AAb positivity			ZnT8 AAb positivity		
	Positive (n = 79)	**Negative (n = 76)**	**p value**	**Positive (n = 20)**	**Negative (n = 135)**	**p value**	**Positive (n = 32)**	**Negative (n = 150)**	**p value**
Age (years)	24.00 [14.03; 55.89]	32.50 [15.04; 56.05]	**0.000***	21.50 [15.04; 35.97]	28.00 [13.88; 55.89]	**0.000***	21.00 [14.05; 34.98]	29.00 [10.05; 56.09]	**0.000***
Age at diagnosis (years)	19.68 ± 8.70	22.28 ± 7.89	0.055	16.40 ± 5.53	21.62 ± 8.56	**0.009***	17.00 ± 6.49	21.48 ± 8.50	**0.006***
Glucose (mmol/L)	9.75 [5.8; 15.3]	8.2 [5.1; 12.9]	0.225	8.3 [5.0; 16.8]	8.7 [5.4;13.2]	0.752	10.85 [7.1; 16.1]	8.4 [5.4; 13.2]	**0.045***
HbA1c (%)	10.73 ± 3.29	10.01 ± 3.27	0.190	10.96 ± 3.46	10.30 ± 3.27	0.431	10.91 ± 3.50	10.56 ± 3.41	0.609
Vitamin D_3 (nmol/L)	63.40 ± 19.34	59.77 ± 17.74	0.226	69.80 ± 20.87	60.41 ± 18.02	**0.035***	61.04 ± 17.32	62.46 ± 19.98	0.710
Male (%)	59.5	52.6	0.389	65.0	54.8	0.392	59.4	53.3	0.533
BMI (kg/m^2)	22.49 [20.96; 25.39]	24.96 [21.72; 29.72]	**0.021***	23.12 [19.41; 27.24]	23.81 [21.34; 28.08]	0.294	22.05 [19.39; 23.86]	23.84 [21.38; 28.73]	**0.003***

Results are presented as median values [lower and upper quartiles] for skewed data and as mean ± standard deviation for non-skewed data, * indicates significant values

3.11 The association of duration of disease with AAb positivity

T1D patients were split into hexiles (1-6) and AAb positivity and duration of disease calculated. Table 3.13 summarises the results obtained for the first and last hexiles (hexile 1 and hexile 6). IA-2 and ZnT8 AAb positivity was significantly higher in patients with a shorter duration of disease compared to those with a longer duration of disease ($p = 0.032$ and $p = 0.008$, respectively). Although it did not reach significance, a similar trend was observed for GAD65 AAb positivity and duration of disease ($p = 0.088$).

Table 3.13: The association of duration of disease with AAb positivity

AAb	Hexile 1			Hexile 6			p value
	Duration (years)	n	AAb positivity (%)	Duration (years)	n	AAb positivity (%)	
GAD65	0.46 ± 0.44	30	63.3	19.23 ± 5.26	27	40.7	0.088
IA-2	0.46 ± 0.44	30	20.0	18.12 ± 5.34	33	3.0	**0.032***
ZnT8	0.46 ± 0.44	37	32.4	18.29 ± 5.25	38	7.9	**0.008***

Results are presented as mean ± standard deviation for non-skewed data, * indicates significant values

3.12 Association of the number of AAbs with clinicopathological variables in T1D patients

The greater the number of AAbs present the younger the age at diagnosis and the shorter the duration of disease. Patients with three AAbs were diagnosed at a much younger age (16.40 ± 6.13 years) than patients who were AAb negative (22.63 ± 8.00 years; $p = 0.020$). Similarly, the greater the number of AAbs present the shorter the duration of disease (3 AAb: 3.33 ± 3.92 years vs. 0 AAb: 9.32 ± 7.01 years; $p = 0.020$). The number of AAbs present did not have any effect on % HbA1c, glucose levels and vitamin D_3 levels (Table 3.14).

Table 3.14: Association of the number of AAbs within diabetic patients with clinicopathological variables

Variable	Number of autoantibodies (n)				p value
	0	1	2	3	
Age at diagnosis (years)	22.63 ± 8.00 (68)	21.04 ± 9.33 (55)	17.20 ± 6.11 (20)	16.40 ± 6.13 (10)	**0.019***
Duration of disease (years)	9.32 ± 7.01 (68)	7.57 ± 7.27 (56)	5.70 ± 3.97 (20)	3.33 ± 3.92 (10)	**0.020***
Glucose (mmol/L)	8.25 [5.15; 12.95] (64)	8.40 [5.40; 13.10] (51)	10.80 [5.80; 16.80] (17)	10.90 [5.80; 17.10] (9)	0.420
HbA1c (%)	9.87 ± 3.14 (63)	10.89 ± 3.34 (52)	10.48 ± 3.67 (18)	10.83 ± 3.22 (10)	0.396
Vitamin D_3 (nmol/L)	59.20 ± 17.73 (69)	62.70 ± 18.69 (56)	64.29 ± 20.70 (20)	66.93 ± 20.23 (10)	0.465

Results are presented as mean ± standard deviation for non-skewed data, * indicates significant values

3.13 Number of AAbs in individuals with a duration of disease less than one year

Within our cohort, of the 22 individuals who were recruited within one year of diagnosis, 19 had been screened for all three AAbs. Of these newly diagnosed T1D patients, 47.4% did not have any AAbs, 36.8% had one AAb and 15.8% had three AAbs (Table 3.15; p = 0.174).

Table 3.15: AAb profiles of T1D participants recruited within 12 months of diagnosis

Number of AAbs	% of newly diagnosed (n)	P value
0	47.4 (9)	0.145
1	36.8 (7)	
2	0.0 (0)	
3	15.8 (3)	

Chapter 4

4 Discussion

T1D is a complex autoimmune disease thought to be the result of the interaction of environmental and genetic factors that result in the destruction of the insulin secreting β-cells of the pancreas. In recent years, interest has focused on vitamin D_3 and the role this hormone may play in the aetiology of T1D. Low levels of vitamin D_3 have been shown to be associated with T1D in many population groups (Greer et al., 2013, Hypponen et al., 2001). Polymorphisms within the *VDR* gene and vitamin D metabolising enzyme genes are believed to negatively influence vitamin D_3 levels (Garcia et al., 2007, Cooper et al., 2011, Hussein et al., 2012). However, these findings are not consistent across all populations studied. Thus, this study aimed to determine whether lower vitamin D_3 levels and polymorphisms within genes linked to vitamin D_3 function are involved in the development of T1D in the South African black population.

In our study lower vitamin D_3 levels were not associated with the development of T1D. In addition, no association was found between *VDR* and metabolising gene polymorphisms and T1D in the black South African population. The presence of the FokI CC and Taq TT genotype were however associated with lower levels of vitamin D_3. In addition, the greater the number of *VDR* risk alleles present, the lower the vitamin D_3 levels in our cohort. IA-2 and ZnT8 AAb positivity was associated with a younger age at diagnosis and a shorter duration of disease. GAD65 AAb positivity was associated with a 23 fold increased risk of T1D development.

4.1 Clinical and phenotypic characteristics of T1D patients and controls

T1D patients are unable to control their blood glucose levels due to a lack of endogenous insulin production (Atkinson et al., 2014). Therefore, it is not surprising that glucose concentrations were significantly higher in T1D patients compared to controls ($p < 0.001$). BMI was significantly lower in the patient group compared to the control group ($p < 0.001$). It is possible that this difference in BMI may be explained by the patients having significantly lower numbers of female participants than controls

(45.6% vs. 56.9 %, respectively; p = 0.040). It is known that South African females tend to have a higher BMI than their male counterparts (27.1 kg/m^2 and 22.9 kg/m^2, respectively) (Puoane et al., 2002).

The majority of autoimmune diseases show a female bias with 1.5 to 5.5 times more female than male sufferers (Fairweather and Rose, 2004, Cooper and Stroehla, 2003). The ratio of T1D males to females in Europe varies widely with countries like Slovenia having a female predominance while Portugal had a higher number of males (Karvonen et al., 1997). In a European epidemiological study there was a greater male preponderance of T1D especially in individuals diagnosed between the ages of 25 - 29 years of age (Kyvik et al., 2004). Similarly, in a Belgian study on white T1D patients there was a very strong male predominance of disease in T1D patients diagnosed between 20 – 39 years of ages (male : female = 2.4 : 1) (Vandewalle et al., 1993). A similar trend is observed in many African populations where a higher proportion of male T1D patients is seen (Kalk et al., 1993). In Ethiopia (0-78 years) and Nigeria (5-7 year olds) the ratio of males to females with T1D was 2:1 and 3:1, respectively (Alemu et al., 2009, Afoke et al., 1992). In Libyan T1D patients a male predominance was seen among the older patients (15-34 years) while a female predominance was shown in younger patients (0-14 years) (Kadiki et al., 1996). However, in studies carried out on black SA and Sudanese T1D patients no statistical difference in gender was noted (Elamin et al., 1989, Elamin et al., 1992, Kalk et al., 1993). Within our population gender was not significantly associated with risk of developing T1D (54.4% vs. 45.6 %, respectively; p = 0.401), supporting the study by Kalk and colleagues.

4.2 Black T1D patients have an older age at diagnosis

The majority (75.6%) of T1D patients in this cohort were diagnosed at ≥ 15 years of age. The peak age at diagnosis of T1D was between 15 -24 years (42.2% of the T1D patients). In contrast, studies in European populations have shown the peak age of onset to be between 5 – 14 years of age, with the greatest increase in incidence seen in children below 5 years of age (Maahs et al., 2010, Harjutsalo et al., 2008, Atkinson et al., 2014). Studies on T1D patients in Sub-Saharan African (SSA) countries support our data showing an older age of diagnosis than that seen in the white population. A

study by Kalk et al. found the peak age at onset in South African black T1D patients attending CHBAH to be 22 - 23 years of age (Kalk et al., 1993). Similarly, a study by Omar et al. conducted on South African black T1D patients from KwaZulu Natal showed a peak age at onset between 21 and 30 years of age (Omar et al., 1984). Tanzanian populations showed the age of onset in their cohorts to be 15 – 19 years of age (Swai et al., 1990). These findings demonstrate that black Africans have an average age at diagnosis approximately a decade later to that seen in white T1D populations, suggesting an ethnic difference in disease aetiology (Alemu et al., 2009, Kalk et al., 1993).

4.3 Allelic frequencies of *VDR* and vitamin D metabolising enzymes gene SNPs within the Black South African population do not differ from frequencies in other African populations

None of the SNPs investigated in this study deviated significantly from Hardy Weinberg Equilibrium and were thus considered representative of the population. The allelic frequencies found in our population did not differ significantly from those reported by the 1000 genomes project (Appendix F) for the African population (Genomes Project et al., 2015). However, allelic frequencies in our black population were significantly different to those seen in the European and East Asian populations with the exception of the ApaI polymorphism. This polymorphism occurs at a similar frequency in the European population.

4.4 *VDR* and vitamin D_3 metabolising enzyme gene polymorphisms are not associated with T1D in the South African black population

The genotypic and allelic frequencies for the *VDR* and vitamin D metabolising enzyme gene polymorphisms were not significantly different in T1D patients compared to control participants. Thus, in the South African black population these SNPs are not associated with the development of T1D.

4.4.1 *VDR* SNPs

The majority of studies have found the FokI C allele to be the risk allele for the development of T1D. The C allele was shown to be the risk allele in west Asia, western Spain, Japan, Iran and Korea (Wang et al., 2014, Zemunik et al., 2005, Ban et al., 2001, Bonakdaran et al., 2012, Cheon et al., 2015). In contrast a study on the Dalmatian population showed the FokI T allele to be associated with the development of T1D. Results are not consistent between studies, even within the same population group as illustrated by the studies by Mohammadnejad et al. and Bonakdaran et al. where Mohammadnejad et al. failed to confirm the association of the homozygous CC genotype with the development of T1D as seen by Bonakdaran et al. in the Iranian population (Mohammadnejad et al., 2012, Bonakdaran et al., 2012). Studies in two distinct Spanish populations namely Navarra and Barcelona, showed conflicting results for the association of the FokI polymorphism with T1D. In the Navarran population the FokI TT genotype was lower in the T1D group compared to controls, whereas no association was seen in the Barcelonian population (Zemunik et al., 2005). Similarly, no associations were seen with the FokI polymorphism and T1D in Saudi and Finnish populations (El-Beshbishy et al., 2015, Turpeinen et al., 2003).

The BsmI A allele has been associated with T1D in Asian populations (Wang et al., 2014, Chang et al., 2000, Cheon et al., 2015). Similarly, a study in the Chilean population found that the A allele was the risk allele for the development of T1D (Garcia et al., 2007). Despite numerous studies demonstrating the association of the BsmI A allele with T1D within different ethnic groups, the associations have not been replicated in all studies. No association was found with either BsmI allele in a cohort of pregnant T1D Saudi women (El-Beshbishy et al., 2015). This lack of association for BsmI was also seen within the Finnish and Iranian populations (Turpeinen et al., 2003, Mohammadnejad et al., 2012). Furthermore, no association was seen between the BsmI polymorphism and T1D in studies on the Australian, British and German populations (Pani et al., 2000, Greer et al., 2013, Nejentsev et al., 2004).

The ApaI A allele was found to be the risk allele for the development of T1D in studies in the Taiwanese and Egyptian populations (Kamel et al., 2014, Chang et al., 2000). However, no association between ApaI genotype and the development of T1D was

found in Finnish, Iranian and Chilean populations (Turpeinen et al., 2003, Mohammadnejad et al., 2012, Garcia et al., 2007).

Studies on the TaqI polymorphism and its association with T1D also show contradictory results. In Egyptians the T allele was found to be the risk allele associated with TID (Kamel et al., 2014). The T allele was also shown to be the risk allele in the Khorasan province of Iran (Bonakdaran et al., 2012). Conversely, the T allele was found to be protective in the Iranian population (Mohammadnejad et al., 2012). Depending on the study, both the T allele and the C allele have been shown to be protective against the development of T1D (Kamel et al., 2014, Bonakdaran et al., 2012, Mohammadnejad et al., 2012). Additional studies in the Taiwanese and Chilean studies have shown no association with either allele or the development of T1D (Chang et al., 2000, Garcia et al., 2007).

To our knowledge, no studies have previously looked at the association of the four *VDR* polymorphisms with T1D in the South African black population. It is clear that conflicting data exists regarding the association of *VDR* SNPs and T1D. These differences in results could be as a result of linkage disequilibrium between risk/ non risk alleles and the *VDR* gene. It is possible that lack of association within our population is due to ethnic differences and further studies within other South African ethnic groups are needed to confirm this.

4.4.2 Vitamin D metabolising enzyme gene SNPs

We found no association between the metabolising gene SNPs studied and T1D in the South African black population. A pilot study conducted in Germany similarly found no association between the *CYP2R1* and *CYP27B1* genes and T1D (Rose et al., 2013). Similar results were obtained in a Polish study where no significant differences were found in *CYP27B1* allelic or genotypic frequencies between T1D patients and healthy controls (Fichna et al., 2010).

These results are contradictory to those conducted in Egyptian, German and British populations where they showed that the *CYP2R1* and *CYP27B1* G alleles were

associated with T1D (Bailey et al., 2007, Cooper et al., 2011, Hussein et al., 2012, Lopez et al., 2004)

It is possible that due to a unique genetic makeup in our population, we failed to show an association.

4.5 Vitamin D_3 levels are not associated with T1D in the South African Black Population

Epidemiological studies have shown large geographical variation in the incidence of T1D. Countries in the Northern hemisphere with higher degrees of latitude correlate with a higher incidence of T1D (Mutlu et al., 2011, Mohr et al., 2008, Liu et al., 2015). At higher latitudes there is decreased sun exposure and thus less UVB rays available to initiate the synthesis of vitamin D_3 within the skin, resulting in decreased vitamin D_3 levels (Rose et al., 2013, Chakhtoura and Azar, 2013). Lower vitamin D_3 levels have previously been associated with the development of T1D (Chakhtoura and Azar, 2013, Mohr et al., 2008).

Vitamin D_3 acts together with RXR and VDR as a messenger to prompt a switch from the Th1 to the Th2 pathway. Within the nucleus, this complex leads to the activation or suppression of transcription of pro/anti-inflammatory cytokines (Kongsbak et al., 2013, Baeke et al., 2010, Prietl et al., 2013). When vitamin D_3 levels are sufficient, the Th2 pathway is favoured and thus β-cells are protected (Altieri et al., 2017, Kamen and Tangpricha, 2010).

There was no significant difference in vitamin D_3 levels between patients and controls when analysing the data using a t-test. This result remained unchanged after adjusting for skin exposure, time outdoors, age, genotype, BMI and season in which the participant was recruited. This is in contrast to studies, in different population groups, that have shown a relationship between low levels of vitamin D_3 and T1D (Chakhtoura and Azar, 2013, Bin-Abbas *et al*., 2011).

In our cohort, the majority of individuals (73.5%) did not have sufficient levels of vitamin D_3. Black South Africans have previously been shown to have low vitamin D_3 levels

despite the sunny climate (Lategan et al., 2016). Possible causes of this insufficiency include, inactivity, smoking, high alcohol consumption and dietary choices (Green et al., 2015). It is possible that within our population, the difference in vitamin D_3 levels between T1D patients and controls are small due to the prevailing vitamin D_3 deficiency in the population. Thus, it would be interesting to determine whether differences in vitamin D_3 levels would be seen in a vitamin D_3 sufficient population.

4.5.1 Vitamin D_3 and seasonal variation

Within our cohort we found that season of sampling was associated with vitamin D_3 levels. Vitamin D_3 levels of individuals sampled in winter were on average 18.20 nmol/L lower that those sampled in summer. Similarly, individuals sampled in spring had on average 7.66 nmol/L less vitamin D_3 than those sampled in summer (Table 3.11). This result may be explained by decreased skin exposure in the winter months due to warmer clothes, as well as shorter days. During spring vitamin D_3 levels would begin to rise due to increased sun exposure, however levels would not be equivalent to those observed in the summer months due to the longer duration and intensity of skin exposure.

Seasonal variations within vitamin D_3 levels are well established (Greer et al., 2013, Chakhtoura and Azar, 2013). Interestingly, seasonal patterns of T1D onset have also been observed with the majority of new cases presenting in the autumn/ winter months (Greer *et al*., 2013, Atkinson *et al*., 2014). Large amounts of melanin in the epidermal layer of individuals causing darker skin types may have reduced the ability of the skin to produce vitamin D (Nair and Maseeh, 2012). In addition, offspring born during spring and winter months tend to be at an increased risk of developing T1D (Simmons and Michels, 2015). Unfortunately, within our cohort, we did not have sufficient information relating to month of diagnosis to determine whether our population follows this trend. Within our cohort, we found seasonal variation in vitamin D_3 levels with the highest levels seen in the summer months and the lowest levels seen in the winter months.

4.5.2 Vitamin D_3 and gender

The male population in our cohort had significantly higher vitamin D_3 levels than the female cohort ($p = 0.009$; Table 3.11). Similar results were seen with a study by Verdoia et al. where females showed lower vitamin D_3 levels than their male counterparts ($p = 0.007$) (Verdoia et al., 2015). In contrast, a Swedish study showed that vitamin D_3 levels in male diabetic patients were significantly lower than female diabetic patients at diagnosis ($p < 0.0001$) (Littorin et al., 2006). Similar results were obtained in an Asian Indian study where males showed lower vitamin D_3 levels than females ($p < 0.02$).

The lower vitamin D_3 levels seen in females in our study may be due to their higher BMI. It is well known that South African black women have a higher BMI than the male population (Puoane et al., 2002). This finding was replicated in our study with males having a lower BMI than females (23.30 [14.99; 42.85] vs. 26.65 [16.82; 46.09]; $p < 0.001$). It has been shown that vitamin D_3 levels correlate negatively with BMI and decline with increasing obesity (Baradaran et al., 2012). It is hypothesised that vitamin D_3 is sequestered within the adipocytes thus preventing it from being available in circulation (Kavaric et al., 2013). In addition, obese individuals may not get enough vitamin D_3 due to a lack of sunlight exposure as a result of decreased mobility or covered clothing habits (Saneei et al., 2013).

Therefore, the discrepancy in vitamin D_3 levels between genders seen in our study could be explained by the lower BMI of males vs. females. In addition, differences in social activities such as spending more time outdoor (such as playing a sport) or outdoor work could result in higher levels of vitamin D_3 observed in the men.

4.6 The FokI CC genotype is associated with lower levels of vitamin D_3

Individuals homozygous for the FokI C allele had significantly lower vitamin D_3 levels ($p = 0.009$) compared to participants with the T allele. This significance remained ($p = 0.013$) when controlling for BMI, gender, season, time outdoors, skin exposure, diabetic status and age. Individuals with the CC genotype had 5.34 nmol/L lower

vitamin D_3 levels than those with the TC and TT genotypes. However, this change in vitamin D_3 levels may be too small to have a clinical effect, and thus does not predispose individuals to T1D in our population.

The FokI polymorphism results in a T to C substitution leading to a three amino acid shorter VDR protein (Jurutka et al., 2000, Uitterlinden et al., 2004a). The shorter protein is 1.7 fold more active than the longer variant (T allele) (Uitterlinden et al., 2004a). In addition, the presence of the short variant results in increased expression of pro-inflammatory cytokine genes, under the transcriptional control of NFAT and NF-κB, resulting in β-cell death (van Etten et al, 2007). It is possible that the increased cytokine transcription occurs through the same mechanism as seen in bone mineralisation via the transcription factor, inducing basal transcription factor IIB (TFIIB) (Jurutka et al., 2000). The FokI polymorphism resides in a region (the activation function 1-like domain (AF-1) in the N-terminal of the zinc fingers) known to bind to TFIIB. Due to the differential interaction of the short form of VDR with TFIIB, more potent transcriptional activity occurs (Jurutka et al., 2000). The AF-1 domain may also be involved in binding to NFAT and NF-κB. Vitamin D_3 inhibits the proliferation of Th1 cells modulating the immune response (Jurutka et al., 2000, Moran-Auth et al., 2015).

4.7 The TaqI TT genotype association with higher levels of vitamin D_3

When performing a t-test, the TaqI TT genotype was found to be associated with significantly higher levels of vitamin D_3 ($p = 0.047$). However, when controlling for BMI, gender, season, time outdoors, skin exposure, diabetic status and age the association disappeared ($p = 0.060$). It is possible that if a larger cohort was studied this may reach significance.

No association between vitamin D_3 levels and the BsmI and ApaI polymorphisms were found in our study. Most studies looked at the association of the *VDR* polymorphisms with the development of T1D however, the effect of *VDR* polymorphisms on serum vitamin D_3 concentration, to our knowledge, have not been previously investigated.

4.8 Vitamin D metabolising gene polymorphisms had no effect on vitamin D_3 levels

Within our cohort, we found no association with metabolising enzyme gene polymorphisms and circulating levels of vitamin D_3. In other studies, *CYP2R1* polymorphisms have been associated with lower vitamin D_3 levels. Patients with the GG or GA genotype in the German population had lower vitamin D_3 levels compared to control participants (Ramos-Lopez et al., 2007a, Cooper et al., 2011). Similarly, diabetic patients in the Egyptian population with the *CYP2R1* GG genotype had lower levels of vitamin D_3 to those with AA genotype (Hussein et al., 2012).

Egyptian individuals with the *CYP27B1* GG genotype had lower vitamin D_3 levels (Hussein et al., 2012). The presence of the *CYP27B1* G allele results in reduced *CYP27B1* mRNA levels. This causes a decrease in the amount of active 1α-hydroxylase and thus decreased conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$, and ultimately lower serum vitamin D_3 levels (Ramos-Lopez et al., 2007b, Moran-Auth et al., 2013). No association between the *CYP27B1* genes, T1D and vitamin D_3 levels were seen in a German study (Rose et al., 2013).

4.9 The effect of the *VDR* risk alleles on vitamin D_3 levels is additive

The *VDR* risk alleles in the study cohort were A, C, A and C for BsmI, FokI, ApaI and TaqI respectively. These risk alleles were based on associations seen in other studies (Table 3.8). Individuals with 7-8 of the *VDR* risk alleles had significantly lower vitamin D_3 levels. Interestingly, when the *VDR* risk alleles in the patient group were compared to vitamin D_3 levels, this significance was lost ($p = 0.051$) indicating that there may be other factors at play in the T1D group.

4.10 Autoantibody frequencies in black South African T1D patients

The appearance of AAbs to GAD65, IA-2, ZnT8 or insulin are currently the most widely used markers for the prediction of T1D. GAD65 AAbs are the most prevalent of the T1D associated AAbs followed by IA-2 and ZnT8 AAbs. In general, GAD65 AAbs are

the most stable and persist throughout the disease, while IA-2 and ZnT8 AAb frequencies decrease with increasing duration of the disease (Kawasaki et al., 2011a, Richardson et al., 2013). The measurement of IAA are not useful following the initiation of insulin therapy as antibodies develop to the exogeneously administered insulin (Sutton et al., 1988). The number of AAbs, rather than the order in which they appear or the titres of an individual AAb, are predictive of an individual's risk of developing T1D (Pihoker et al., 2005, Verge et al., 1996).

Within our cohort GAD65 AAbs were the most prevalent (51%) of the three autoantibodies studied and GAD65 AAb positivity was associated with a 23 fold increased risk of T1D. ZnT8 AAbs were found in 17.6% of patients while only 12.9% of T1D patients were IA-2 AAb positive. Due to the fact that all our patients were taking insulin, we did not measure IAA. These AAbs appear to be more prevalent in white populations with frequencies ranging between 70 - 80%, 32 - 75% and 60 - 80% for GAD65, IA-2 and ZnT8 AAbs in newly diagnosed T1D patients, respectively (Notkins and Lernmark, Winter, Wenzlau et al 2007, Andersson et al, 2011).

Within African American T1D patients, GAD65 AAbs have been found at a frequency of 54 - 75% while IA-2 AAbs were found at a frequency of 38 - 51% (Libman et al., 2003, Leech et al., 1995, Lipton et al., 2011). A Tunisian study on newly diagnosed diabetic patients found similar frequencies for GAD65 (65.1%) and IA-2 AAbs (43%) to those seen in the African American population (Fakhfakh et al, 2008). In contrast, a Cameroonian study, showed only 34% GAD65 AAb positivity and 6.4% IA-2 AAb positivity (Hawa et al, 2006). A study on Somalian T1D patients living in Minnesota found a ZnT8 AAb prevalence of 26% (Sunni et al, 2017). In Algeria, the frequency for ZnT8 AAbs was reported as 46.3% (Lounici Boudiaf et al., 2018). As the Algerian population consists of 99% Arab ethnicity and only 15% of Somalia is black African, we are unable to use this data as an accurate comparison with our ZnT8 AAb data (Central Intelligence Agency, 2018). In non-Hispanic blacks, the ZnT8 AAb frequency was higher (57.9%) than that observed in our study (Wenzlau et al., 2015).

A study by Panz et al. showed that 44% of black South African T1D patients were GAD65 AAb positive (Panz et al., 2000). Similarly, another South African study by Rheeder et al. showed that GAD65 AAbs were found in 33% of black patients vs. 67%

of the white patients (Rheeder et al., 2001). To our knowledge, no studies have looked at the frequency of IA-2 or ZnT8 AAbs in South African black T1D patients.

Our population showed similar frequencies for IA-2 AAb positivity to that seen in a Tanzanian (12.8%) and Cameroonian study (6.4%) (Lutale et al., 2007, Hawa et al., 2006). However, they differed significantly to a study in Tunisia (43%). It is possible that this discrepancy is due to the Tunisian study being reported in newly diagnosed T1D patients and it is well documented that the frequency of IA-2 AAbs decline with increased disease duration. GAD65 AAb positivity from studies conducted in black South African and SSA T1D patients were similar to that seen in our study.

A number of factors can influence the differences observed in AAb positivity. These include the age of the patient at diagnosis, duration of disease and ethnicity (as seen above).

4.10.1 AAb positivity and age at diagnosis

IA-2 and ZnT8 AAb frequencies decline with increasing age at diagnosis (Gorus et al., 1997, Verkauskiene et al., 2016, Wenzlau et al., 2007). In contrast, a number of studies have shown that GAD65 AAb positivity is associated with an older age at diagnosis (Thai et al., 1997, Verkauskiene et al., 2016, Vandewalle et al., 1995). In our study, IA-2 and ZnT8 autoantibody positivity was associated with a younger age at diagnosis. A similar trend was observed for GAD65 AAb positive patients, however this did not reach significance. In addition, our T1D patients had an earlier age of diagnosis with an increasing number of AAbs (16.40 ± 6.13 vs. 22.63 ± 8.00 years, for 3 vs. 0 AAbs respectively; $p = 0.019$).

Interestingly, several studies have shown that IA-2 AAb positivity is associated with the age of the patient rather than the age at diagnosis (Cheng et al., 2018, Gorus et al., 1997, Tridgell et al., 2011). The majority of our South African black T1D patients were diagnosed between the ages of 15 – 24 years. Therefore, it is possible that the lower frequency of IA-2 AAb positivity seen within our population is as a result of the later age at which this population develops T1D.

4.10.2 AAb positivity and duration of disease

IA-2 and ZnT8 AAb frequencies and titres decline with increasing duration of disease (Kawasaki et al., 2011b, Cheng et al., 2018, Wenzlau et al., 2015). GAD65 AAb are found in patients of all age groups and remain persistently positive throughout the duration of disease even though titres may decline slightly (Fida et al., 2001, Thai et al., 1997). However, this trend is not always seen (Verkauskiene et al., 2016).

In our study, IA-2 and ZnT8 AAb positivity was significantly higher in patients with a shorter duration of disease ($p = 0.032$ and $p = 0.008$, respectively). GAD65 AAb positivity showed a similar trend with disease duration, however this did not reach significance ($p = 0.088$).

It is possible that the decline in AAb positivity seen over time is due to continuous destruction of β-cells (Cheng et al., 2018). As the β-cells are destroyed, fewer antigens are presented to B cells and hence fewer AAb are produced.

4.10.3 Number of autoantibodies and T1D positivity

Many studies show that multiple AAbs are found in their highest titres at onset of disease (Ziegler et al., 2012). In white populations, only 10-15% of T1D patients do not present with any AAbs. In contrast, studies in African American T1D patients show that 50-60% are AAb negative (Babu et al, 2001; Tiberti et al, 2000).

Within our study we found that 6.5% of the patients were positive for all three autoantibodies, 13.1% were positive for two autoantibodies, 35.9% were positive for one autoantibody and 44.4% were autoantibody negative (data not shown). In contrast, when looking at the newly diagnosed patients (12.1% of the total cohort with < 1 year duration), 15.8% had three AAbs present, 36.8% had one AAb and the majority of newly diagnosed patients (47.4%) had no AAbs. In Africa the percentage of individuals positive for more than one AAb ranged from 8.5 to 81.4% (Lutale et al., 2007, Fakhfakh et al., 2008). Within white populations, 90% of newly diagnosed patients have one or more AAbs (Ziegler et al., 2013, Nokoff and Rewers, 2013). Thus, there seem to be ethnic differences in the number of AAb present in an individual.

The presence of AAbs can be used to confirm diabetes status in South African patients; however, the lack of AAbs is not sufficient to exclude a T1D diagnosis.

4.11 Limitations of the study

It is possible that the n number for the study cohort may not have been large enough to see all effects. In this study we did not determine the effects of haplotypes on diabetic status.

GAD65 AAbs are found in adult T1D patients that have been diagnosed later in life. These patients are referred to as latent autoimmune diabetes of adults (LADA) (Atkinson and Eisenbarth, 2001). Within the SA black population, we see a later age of onset for diagnosed T1D diabetics, many of whom present with GAD65 AAbs. Thus it is important to consider the possibility that some of the late onset patients may in fact be LADA patients and not true T1D patients. However, the current patients all required insulin within six months of diagnosis and are therefore most probably not misclassified. Furthermore, not all participants were screened for all AAbs and therefore the frequencies reported may not be accurate.

Chapter 5

5 Conclusion

In conclusion, the *VDR* and vitamin D metabolising enzyme gene polymorphisms were not associated with the development of T1D within our black South African population. The presence of the FokI CC genotype was associated with lower levels of vitamin D_3. In our study we showed that there was an inversely proportional relationship between the number of *VDR* risk alleles and vitamin D_3 levels. Interestingly, despite this association, the presence of risk alleles did not predispose to T1D, implying that vitamin D_3 may not impact on T1D disease progression in this cohort.

The age at diagnosis within our population was found to be at least a decade later than that seen in white populations with 75.6% of patients being diagnosed at ≥ 15 years of age. Within our cohort 12.1 % were recruited within the first year of diagnosis. Of the newly diagnosed individuals, 47.4% were AAb negative, which is a much higher frequency than that found in white populations, indicating that AAb negativity within our population is not sufficient to exclude the diagnosis of T1D. Black South African individuals who are AAb positive are more likely to develop T1D at an earlier age than those who are AAb negative. In addition, the greater the number of AAbs for which an individual is positive, the younger the age at diagnosis.

Black South African individuals who are GAD65 AAb positive are 23 fold more likely to develop T1D than GAD65 negative patients. IA-2 and ZnT8 AAbs within our population decline over the duration of disease, whereas GAD65 AAbs remained prevalent throughout disease progression.

Thus, our black South African population may have a unique disease aetiology which warrants further investigation.

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7 Appendices

7.1 Appendix A: Stock solutions and dilution preparations

TBE Buffer (10x)

108 g Tris(hydroxymethyl)aminomethane

55 g Boric acid

7.4 g Ethylenediaminetetra-acetic acid (EDTA)

Make up to 1 litre with distilled water (dH_2O).

TBE Buffer (1x)

100 ml TBE

900 ml dH_2O

Agarose gel (1%)

1g agarose

100 ml 1 x TBE

Microwave for 2 minutes, cool slightly and add 1µl GelRed (10 000x) per 100 ml of gel.

Agarose gel (2%)

2g agarose

100 ml 1 x TBE

Microwave for 2 minutes, cool slightly and add 1µl GelRed (10 000x) per 100 ml of gel.

Bromophenol blue loading dye

0.1% Bromophenol blue

10ml 0.5M EDTA

25 ml Glycerol

Make up to 50 ml with dH_2O.

0.5M EDTA

18.61g EDTA

Make up to 100ml with dH_2O.

7.2 Appendix B: Questionnaire

Demographics

Study number
Date of visit (dd/mm/yyyy):

Biogram

Date of birth (dd/mm/yyyy):
Gender: M / F
Race:
Telephone number of self or best possible contact:

Family history of diabetes:

On Mothers side:
On Fathers side:
Of Siblings:

Risk Factors:

Smoking: Y / N / Ex / U *Comments:*	(ex > 1yr stopped)
Snuff user: Y / N / Ex / U *Comments:*	(ex > 1yr stopped)

Random Capillary Glucose (from file):
HbA1c within last 4 months:
Blood pressure (mmHg):
Weight (kg):
Height (cm):
Waist circumference (cm):
Hip circumference (cm):
Acanthosis Nigricans: Y / N / U Where:

DM year of diagnosis:
Clinical judgement on type of DM: 1 / 2 T1 = age of diagnosis before 30 (and on insulin within 1 yr of dx) or on insulin within 1 yr of diagnosis regardless of age of diagnosis
Hypertension on treatment: Y / N / U Year of diagnosis:

History:

1. Presentation at time of diagnosis	
DKA or severe hyperglycemia requiring hospitalization	Y / N / U
Coincidental finding, e.g. at surgery or other illness	Y / N / U
Symptoms such as polyuria/polydipsia causing patient to visit a health care facility	Y / N / U
Weight loss at time of diagnosis	Y / N / U
2. Insulin started at time of diagnosis?	Y / N / U
3. If on insulin now, was it started within 1 year of diagnosis?	Y / N / U
4.Complications	
Macro: MI Stroke Revascularization Amputation	
Micro: Lasered Nephropathy Neuropathy)	

Medication (from list)

Insulin
Oral medication
Others

Supplementation

1.Are you on vitamin D supplements	Y / N / U

Other diseases

1.
2.
3.

SUN EXPOSURE

Please circle which best describes your main occupation:

Mainly indoors
Half indoors and half out doors
Mainly outdoors

[1]

	Time Outdoors			Amount of Skin Exposed			
	<5 min	5-30 min	>30min	Hands and face	Hands, face, arms	Hands, face, legs	Bathing suit
Monday	0	1	2	1	2	3	4
Tuesday	0	1	2	1	2	3	4
Wednesday	0	1	2	1	2	3	4
Thursday	0	1	2	1	2	3	4
Friday	0	1	2	1	2	3	4
Saturday	0	1	2	1	2	3	4
Sunday	0	1	2	1	2	3	4

Estimates of percentage of BSA exposed based on the "rule of nines" and clothing worn[2]

Body region[3]	Clothing type	% BSA exposed
Head	Nothing	4
	Beanie/bike helmet	3
	Baseball cap	2
	Cowboy hat	1
	Nothing	47
Torso/arms	Bikini top/sports bra	42
	Tank top	18
	Tee shirt	10
	Quarter length shirt	3
	Long sleeves	0
Legs	Bikini bottom	38
	Short shorts/ Skirt	24
	Knee-length shorts/skirt	8
	Full-length pants	0
Feet	Nothing	2
	Sandals	1.5
	Shoes	0

[1] Sun exposure questionnaire predicts circulating 25-hydroxyvitamin D concentrations in Caucasian hospital workers in southern Italy. Hanwell HE, Vieth R, Cole DE, Scillitani A, Modoni S, Frusciante V, Ritrovato G, Chiodini I, Minisola S, Carnevale V. *J Steroid Biochem Mol Biol.* 2010 Jul;121(1-2):334-7.

[2] From: Vitamin D Intake Needed to Maintain Target Serum 25-Hydroxyvitamin D Concentrations in Participants with Low Sun Exposure and Dark Skin Pigmentation Is Substantially Higher Than Current Recommendations. Hall LM, Kimlin MG, Aronov PA, Hammock BD, Slusser JR, Woodhouse LR, Stephensen CB. *J Nutr*. 2010 Mar;140(3):542-50.

[3] Hands contributed 4% of exposed BSA (0% if gloves were worn) and the neck 2% (0% if a scarf was worn).

7.3 Appendix C: Ethics certificates

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Ms Sureka Bhola et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180334

NAME: (Principal Investigator)	Ms Sureka Bhola et al
DEPARTMENT:	Chemical Pathology
PROJECT TITLE:	The Role of Vitamin D in the Aetiology of Type 1 Diabetes Mellitus in the South African Black Population
DATE CONSIDERED:	06/04/2018
DECISION:	Approved unconditionally
CONDITIONS:	A Sub-study under Primary Study M150885 Dr Carolyn Padoa
SUPERVISOR:	Dr Carolyn Padoa
APPROVED BY:	Professor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	10/05/2018

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

R14/49 Dr Carolyn Padoa

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150885

NAME: (Principal Investigator)	Dr Carolyn Padoa
DEPARTMENT:	Chemical Pathology
PROJECT TITLE:	Phenotypic and Genotypic Characterisation of Patients Diagnosed with Diabetes Mellitus before the Age of 40 in South Africa (Previously M10978)
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal (2015 - 2020)
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	09/09/2015

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Dear Dr Carolyn Padoa

DATE OF COMMITTEE MEETING:	**10 April 2014**
PROJECT TITLE:	**Phenotypic and genotypic characterization of patients diagnosed with diabetes mellitus before the age of 40 in South Africa (NRR) Donors**
DECISION OF THE COMMITTEE:	**Approved**
CLEARANCE CERTIFICATE NO:	**2014/19**

- Execution of the study must be compliant with applicable guidelines and policies.
- Any amendment, extension or other modifications to the protocol must be submitted to this Ethics Committee for approval prior to implementation.
- The Committee must be informed of any serious adverse event, planned and unplanned termination of the study.
- A progress report should be submitted yearly for long-term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the SANBS HREC clearance certificate number on all future correspondence on this study to the HREC secretariat.
- This approval is valid for 5 years from the date stated above.

COMMITTEE GUIDANCE DOCUMENTS:

- International Conference on Harmonization (ICH) Good Clinical Practices (GCP) Guideline (ICH, 1996), Ethics in Health Research: Principles, Structures and Procedures (SA Department of Health, 2004); Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa (SA Department of Health, 2006); Ethical Principles for Medical Research Involving Human: Declaration of Helsinki (World Medical Association, 2013); Reviewing Clinical trials: A Guide For Ethics Committees (Karlberg and Speers, 2010)

27 February 2015

CHAIRPERSON: Prof J.N. Mahlangu **DATE**

7.4 Appendix D: PCR gel images

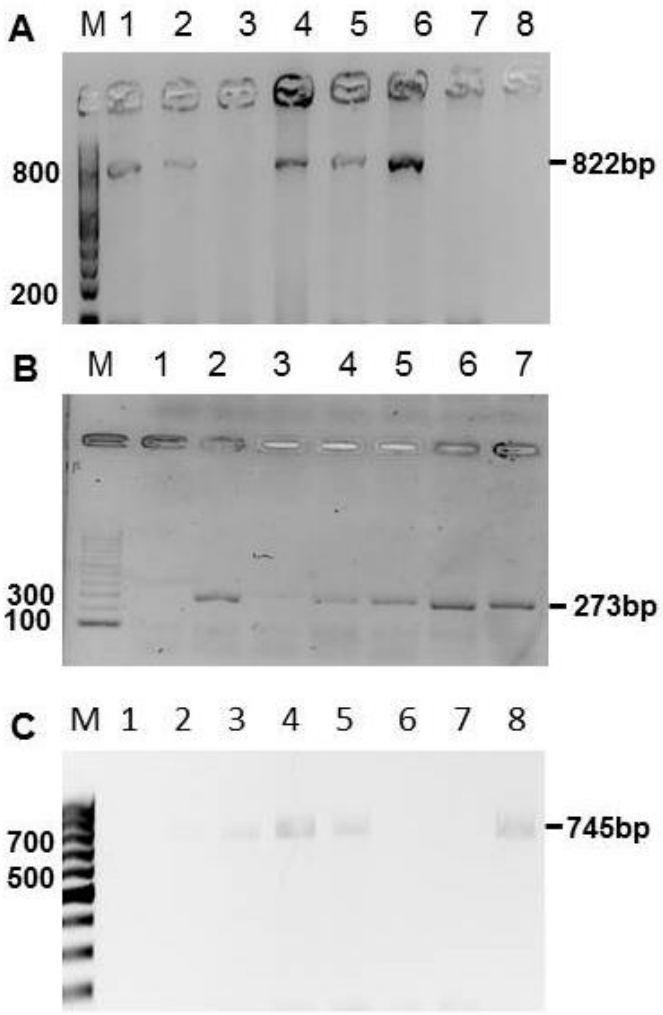


Figure D.1: PCR amplification of the *VDR* gene flanking the BsmI (A), FokI (B) and ApaTaq (C) polymorphisms.

(A) BsmI PCR gel: Lane M: 100bp DNA ladder; Lane (1, 2, 4-6): 822bp PCR products; Lane (3, 7): no amplification; Lane 8: negative control.

(B) FokI PCR gel: Lane M: 100bp DNA ladder. Lane (1): negative control; Lane (2, 4-7): 273 bp PCR products; Lane (3): no amplification.

(C) ApaTaq PCR gel: Lane M: 100bp DNA ladder; Lane (1): negative control; Lane (2-5, 8): 745bp PCR products; Lane (6-7): no amplification.

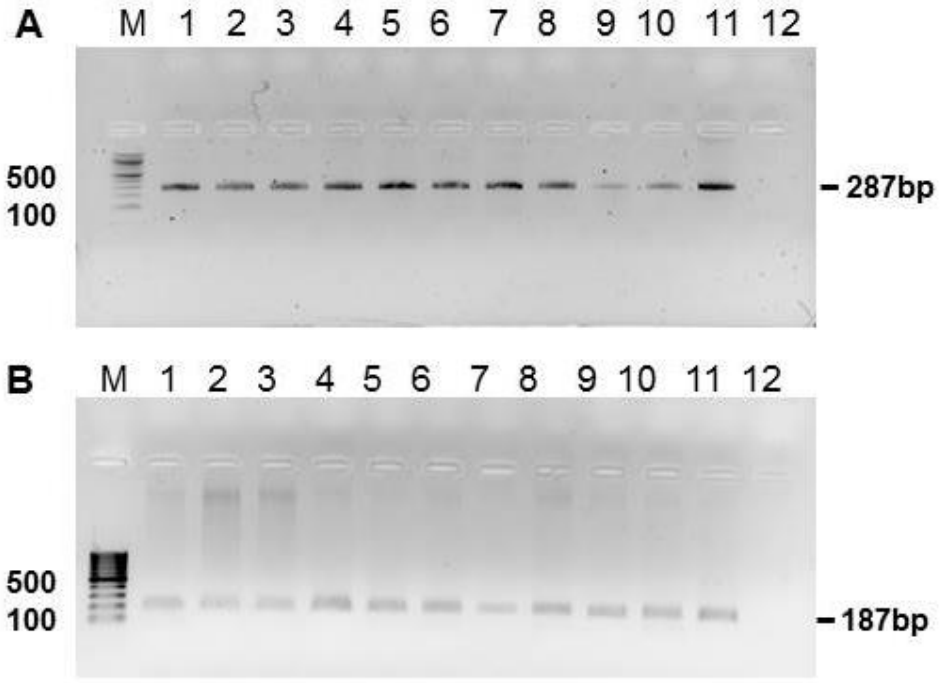


Figure D.2: PCR amplification of the vitamin D metabolising enzyme genes flanking the *CYP2R1* (A) and *CYP27B1* (B) polymorphisms.

(A) *CYP2R1* PCR gel: Lane M: 100bp DNA ladder; Lane (1-11): 287bp PCR products; Lane 12: negative control.

(B) *CYP27B1* PCR gel:. Lane M: 100bp DNA ladder; Lane (1-11): 187bp PCR products; Lane 12: negative control.

7.5 Appendix E: Hardy Weinberg Equilibrium

Genotype model	Participants (n)	Chi^2 (χ^2)	p Value
BsmI (rs1544410)		**1.15**	**0.68**
GG	0.65 (205)		
GA	0.30 (96)		
AA	0.05 (16)		
FokI (rs2228570)		**0.027**	**0.87**
TT	0.04 (12)		
CT	0.30 (100)		
CC	0.66 (221)		
ApaI (rs7975232)		**0.17**	**0.68**
CC	0.11 (37)		
CA	0.43 (142)		
AA	0.46 (151)		
TaqI (rs731236)		**0.028**	**0.87**
TT	0.65 (212)		
TC	0.31 (102)		
CC	0.04 (13)		
CYP2R1 **(rs10741657)**		**1.16**	**0.28**
GG	0.65 (213)		
GA	0.33 (108)		
AA	0.03 (9)		
CYP27B1 **(rs10877012)**		**0.17**	**0.68**
GG	0.83 (276)		
GT	0.17 (55)		
TT	0.00 (0)		

7.6 **Appendix F:** Comparison of *VDR* and metabolising enzyme gene polymorphism frequencies between our cohort and populations from the 1000 genomes project

SNP	**Our Study**	**African**	**p value**	**European**	**p value**	**East Asian**	**p value**
BsmI (G allele)	0.80	0.73	0.156	0.60	<0.001*	0.94	0.001*
FokI (T allele)	0.19	0.19	0.927	0.38	<0.001*	0.42	<0.001*
ApaI (C allele)	0.33	0.36	0.593	0.45	0.022*	0.71	<0.001*
TaqI (T allele)	0.80	0.71	0.042	0.60	<0.001*	0.93	0.004*
***CYP2R1* (G allele)**	0.81	0.78	0.583	0.62	<0.001*	0.68	0.005*
***CYP27B1* (G allele)**	0.92	0.92	0.925	0.68	<0.001*	0.36	<0.001*

Data obtained from the 1000 genomes project (Genomes Project et al., 2015); * represents significance $p < 0.05$ when compared to our study.

Based on the frequency of the minor allele for all the SNPs studied, our sample size (calculated with Sample Size Calculator) was large enough and, therefore, sufficiently powered, with the exception of ApaI. In addition, when comparing the n number of this study to those in the literature, the majority had similar, if not smaller, samples sizes than ours.

7.7 Appendix G: Duration of disease hexiles for individuals screened for GAD65, IA2 and ZnT8 AAbs

	Duration of disease (years)		
Hexile	**GAD65 (n)**	**IA2 (n)**	**ZnT8 (n)**
1	<2 (30)	<2 (30)	<2 (37)
2	2-3 (21)	2-3 (21)	2-3 (26)
3	4-6 (22)	4-6 (22)	4-6 (27)
4	7-9 (29)	7-8 (22)	7-8 (24)
5	10-16 (25)	9-12 (26)	9-12 (29)
6	>13 (27)	>12 (33)	>12 (38)