

https://dspace.org/

The role of vitamin D in the aetiology of type 1 diabetes in the South African Indian and White population

Malaza, Nompumelelo Lebogang

2019
https://hdl.handle.net/10539/29759

Downloaded from a DSpace repository. https://dspace.org/

The role of vitamin D in the aetiology of type 1 diabetes in the South African Indian and White population

Nompumelelo Lebogang Malaza

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 2019

Declaration

I, Nompumelelo Lebogang Malaza, declare that the project titled “The role of vitamin D in the aetiology of type 1 diabetes in the South African Indian and White population” is my own work. It is being submitted in fulfilment for the degree of Masters of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

Nompumelelo Lebogang Malaza

21st day of August 2019 at the University of the Witwatersrand Medical School

Dedication

I would like to dedicate this dissertation to my mother. Thank you for continuous support and encouragement, much appreciated.

Presentations arising from this study

1. **Malaza NL**, Cave E, Pirie FJ, Motala AA, Crowther NJ, Padoa CJ (2018) The association between vitamin D receptor gene polymorphisms and type 1 diabetes in the South African Indian population. 18th International Congress of Endocrinology (Cape Town)

2. **Malaza NL**, Cave E, Pirie FJ, Motala AA, Crowther NJ, Padoa CJ (2018) The association between vitamin D receptor gene polymorphisms and type 1 diabetes in the South African Indian population. WITS Research Day (Johannesburg)

3. **Malaza NL**, Cave E, Pirie FJ, Motala AA, Crowther NJ, Padoa CJ (2018) The association between vitamin D receptor gene polymorphisms and type 1 diabetes in the South African Indian population. Wits Post Graduate Symposium (Johannesburg)

Abstract

Background: Type 1 diabetes (T1D) is a T-cell mediated autoimmune disease characterised by the presence of autoantibodies to β cell antigens such as insulin, glutamic acid decarboxylase and most recently zinc transporter 8 (ZnT8). The exact aetiology of T1D is not fully understood. Recently, vitamin D has been implicated in the development of the disease. Vitamin D is a steroid hormone with a wide range of biological functions including immunoregulation. Vitamin D exerts its immunomodulatory effects by binding to the vitamin D receptor (VDR) which activates a cascade of downstream signalling events. Vitamin D downregulates the expression of pro-inflammatory cytokines resulting in a shift from the T helper 1 (Th1) pathway (responsible for the destruction of pancreatic β cells) to the anti-inflammatory T helper 2 (Th2) pathway. Thus, vitamin D is believed to be protective against the development of T1D. Four *VDR* gene polymorphisms (*Bsm*I [rs1544410], *Fok*I [rs2228570], *Apa*I [rs7975232] and *Taq*I [rs731236]) have been identified and shown to be associated with the development of T1D in different population groups. In addition, polymorphisms in vitamin D metabolising enzyme genes (*CYP2R1* [rs10741657] and *CYP27B1* [rs10877012]) have been associated with T1D. To our knowledge, there is no data on the association of these polymorphisms in the South African Indian and White populations. Therefore, the 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 South African Indian and White populations. In addition, this study aimed to determine whether circulating vitamin D levels are associated with the development of T1D.

Methods: Indian (83 T1D patients and 88 control participants) and White participants (106 T1D patients and 87 control participants) were recruited from the Gauteng and Durban regions in South Africa. All participants were genotyped by polymerase chain reaction- restriction fragment length polymorphisms (PCR-RFLP) for the *VDR* and vitamin D metabolising enzyme gene polymorphisms. Vitamin D levels were measured using the ClinRep kit on an high pressure liquid chromatography (HPLC) instrument. Zinc transporter 8 (ZnT8) autoantibody positivity was measured in all participants by EuroImmun enzyme linked immunosorbent assay (ELISA). Glucose concentrations were measured with the Cobas 6000 analyser. *VDR* mRNA expression levels were measured by quantitative real time PCR in a subset of the White population.

Results: The median age at diagnosis was 13.0 years with a mean duration of 11.7 years in the Indian T1D cohort. The median age at diagnosis in the White T1D patients was 20.0 years with a mean duration of 21.8 years. ZnT8 autoantibodies were only detected in 21.3% of the Indian T1D and 23.6% of the White T1D patients. In the Indian population, the A alleles of *Bsm*I, *Apa*I and *CYP2R1* polymorphisms showed significantly higher frequencies in T1D patients as compared to controls ($p = 0.023$, $p = 0.009$ and $p < 0.001$, respectively). Indian control participants had significantly fewer *VDR* and vitamin D metabolising enzyme gene risk alleles than T1D patients ($p = 0.027$ and $p = 0.016$, respectively). The majority of the White population (60.2%) and only 28.4% of the Indian population had sufficient levels of vitamin D. In our cohort, low levels of vitamin D were not associated with T1D. In the total study population, Indian ethnicity and *CYP2R1* GG/GA genotype were observed to significantly contribute to lower vitamin D levels ($p < 0.001$ and $p = 0.026$, respectively).

Conclusion: *VDR Bsm*I and *Apa*I polymorphisms and the *CYP2R1* vitamin D metabolising enzyme gene polymorphism are associated with the development of T1D in the South African Indian population. In addition, increasing numbers of risk alleles were associated with T1D in the Indian cohort. Polymorphisms in the VDR gene may alter its structure preventing the binding of vitamin D. Alternatively, it could result in reduced transcription of the VDR. Since vitamin D exerts its immunoregulatory effects through the VDR, this lack of VDR activation may result in the upregulation of pro-inflammatory cytokines, β cell destruction and ultimately the development of T1D. Polymorphisms in the *CYP2R1* gene may lead to reduced activity of the 25 hydroxylase enzyme and hence reduced production of the active vitamin D metabolite. Thus, reduced vitamin D levels will lead to T1D due to failure of the activation of the vitamin D signalling cascade.

Acknowledgments

- I would like thank the following:
- My supervisors Dr C Padoa and Dr E Cave, for their patience, advice and assistance with my laboratory work and the writing of my dissertation. Your support and efforts are sincerely and highly appreciated.
- To nurse Nomsa and the Chemical Pathology registrars, for taking time out of their schedules to draw blood samples for me.
- To Katherine Prigge for integration and interpretation of the vitamin D samples.
- To my fellow colleagues, thank you for your advice in and out of the laboratory.
- To the University of Witwatersrand and Department of Chemical Pathology, for giving me the opportunity to further my studies in a well-equipped and conducive environment.
- To the South African Medical Research Council Internship Scholarship Program for the financial assistance.
- To the NHLS Research trust for funding this study.

Table of Contents

Declaration ii

Dedication iii

Presentations arising from this study iv

Abstract v

Acknowledgments vii

List of abbreviations, symbols and units xii

List of Figures xv

List of Tables xvii

1 Literature Review 1

1.1 Vitamin D 1

1.1.1 Metabolism of vitamin D 1

1.2 Vitamin D as an immune modulator 2

1.3 Vitamin D and its association with disease 4

1.4 Type 1 diabetes 4

1.4.1 Environmental factors 6

1.4.2 Genetic susceptibility 6

1.4.3 The pathogenesis of T1D 7

1.4.4 Autoantibodies 9

1.4.5 The epidemiology of T1D 10

1.5 Vitamin D as an immune modulator 11

1.6 Vitamin D deficiency 13

1.6.1 Vitamin D deficiency and T1D 14

1.7 *VDR* polymorphisms and T1D 14

1.7.1 The *Bsm*I polymorphism 15

1.7.2 The *Fok*I polymorphism 15

1.7.3 The *Apa*I polymorphism 16

1.7.4 The *Taq*I polymorphism 16

1.8 Polymorphisms in the vitamin D metabolising enzyme genes and their association with T1D 16

1.8.1 The *CYP2R1* polymorphism 17

1.8.2 The *CYP27B1* polymorphism 17

1.9 Aim and objectives 18

2 Methods 19
2.1 Participant recruitment 19
2.2 Sample processing 19
2.3 DNA extraction 20
2.4 Genotyping participants for polymorphisms in the *VDR* gene 20
2.4.1 polymerase chain reaction of the *VDR* gene 20
2.4.2 Agarose gel electrophoresis 22
2.4.3 Restriction Fragment Length Polymorphism (RFLP) for genotyping participants for the *VDR* polymorphisms 22
2.5 Genotyping participants for polymorphisms in the vitamin D metabolising enzyme genes 24
2.5.1 Polymerase Chain Reaction of the vitamin D metabolising gene polymorphisms 24
2.5.2 Restriction Fragment Length Polymorphism for genotyping participants for the *CYP2R1* and *CYP27B1* polymorphisms 26
2.6 Gene expression studies for the *VDR* gene 27
2.6.1 Isolation of peripheral blood mononuclear cells 27
2.6.2 RNA extraction 27
2.6.3 DNase digestion 28
2.6.4 cDNA synthesis 28
2.6.5 Quantitative real time PCR 29
2.7 Measurement of serum vitamin D 30
2.7.1 Extraction of vitamin D 31
2.8 Measurement of ZnT8 AAbs 32
2.9 Measurement of serum glucose 32
2.10 Statistical analysis 33
3 Results 34
3.1 Characteristics of the study population 34
3.2 Anthropometric and clinical characteristics of participants in the Indian and White populations 35
3.2.1 Comparison of anthropometric and clinical characteristics between Indian and White T1D patients 38
3.2.2 Distribution of age at diagnosis of T1D participants 39
3.2.3 ZnT8 autoantibody status and clinicopathological variables in T1D patients 39
3.3 Genotyping participants for the *VDR* and vitamin D metabolising enzyme gene polymorphisms 41
3.4 Sanger sequencing of *VDR* and vitamin D metabolising enzyme gene PCR products 42

3.5 Genotypic and allelic frequencies for the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the Indian and White control populations 42
3.5.1 Comparison of allelic frequencies for *VDR* and vitamin D metabolising enzyme gene polymorphisms between the Indian and White South African control cohorts and populations in the 1000 genomes project 45
3.5.2 Genotypic and allelic associations of the *VDR* and vitamin D metabolising enzyme gene polymorphisms with T1D in the study cohort 48
3.6 Association of clinicopathological variables with *VDR* and vitamin D metabolising enzyme gene polymorphisms in the Indian and White T1D participants 53
3.7 The association of increased numbers of *VDR* and vitamin D metabolising enzyme gene polymorphism risk alleles with T1D in Indian and White populations 58
3.7.1 Comparison of clinicopathological variables and the number of *VDR* gene risk alleles in the Indian and White T1D patients 58
3.7.2 Comparison of clinicopathological variables and the number of vitamin D metabolising gene risk alleles in the Indian and White T1D patients 61
3.8 Multivariable linear regression model to determine which variables contribute to vitamin D levels in the total cohort 63
3.9 Comparison of relative *VDR* mRNA expression levels between White T1D patients and control participants 64
4 Discussion 66
4.1 Anthropometric and clinical characteristics of the White and Indian populations 66
4.2 Characteristics of the Indian and White T1D participants 68
4.2.1 Indian T1D patients have a younger age at diagnosis than White T1D patients 68
4.2.2 A low prevalence of ZnT8 AAb positivity in South African Indian and White T1D patients 69
4.2.3 Indian T1D patients have poorer glucose control than White T1D patients 70
4.2.4 Indian T1D patients have lower vitamin D levels than White T1D patients 70
4.3 Genotypic and allelic frequency of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the study population 70
4.3.1 *VDR* polymorphism allelic frequencies in South African Indian and White participants are not significantly different from population matched frequencies in the 1000 Genomes project. 71
4.3.2 The frequency of the *CYP27B1* polymorphism in the White South African population is significantly different to published literature 71
4.4 The association of *VDR* polymorphisms with T1D in the South African Indian and White population 72
4.4.1 The *Bsm*I and *Apa*I *VDR* polymorphisms are associated with T1D in the South African Indian population 72
4.4.2 The *VDR* polymorphisms are not associated with T1D in the South African White population 74

4.5 The association of vitamin D metabolising gene polymorphisms with T1D in the South African Indian and White population 74
4.5.1 The *CYP2R1* polymorphism is associated with T1D in the South African Indian population 74
4.5.2 The *CYP27B1* polymorphism is associated with T1D in the South African White population 75
4.6 *Apa*I genotypes contribute to differences in clinicopathological variables in Indian T1D participants 75
4.7 *VDR* and vitamin D metabolising enzyme gene polymorphisms are not associated with lower levels of vitamin D in South African Indian and White T1D patients 76
4.8 An increasing number of *VDR* and vitamin D metabolising enzyme gene polymorphism risk alleles are associated with T1D in the Indian but not White population 77
4.9 Indian ethnicity and the *CYP2R1* GG genotype contribute to lower vitamin D levels 78
4.10 *VDR* mRNA expression is higher in T1D patients when compared to control participants 79
4.11 Limitations of the study 79
5 Conclusions 81
6 References 82
7 Appendices 99
7.1 Appendix 1: Informed consent and questionnaire 99
7.1.1 Informed consent 99
7.1.2 Questionnaire 102
7.2 Appendix 2: Ethics Certificates 104
7.2.1 Wits Human Ethics Committee certificate 104
7.2.2 SANBS Ethics Committee certificate 105
7.3 Appendix 3: PCR gels 106
7.4 Appendix 4: Chromatograms 107
7.5 Appendix 5: Real time PCR amplification graphs and melt curves 109
7.6 Appendix 6: Turn-it-in report 111

List of abbreviations, symbols and units

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
7-DHC	7-dehydrocholesterol
$25(OH)D_3$	25-Hydroxyvitamin D3
AAb	Autoantibody
ANOVA	Analysis of variance
APC	Antigen presenting cells
AP1	Activator protein 1
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Base pairs
CDE	Centre of Diabetes and Endocrinology
cDNA	Complementary deoxyribonucleic acids
CTL	Cytotoxic T lymphocytes
DBD	DNA binding domain
DNA	Deoxyribonucleic acids
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fas L	Fas ligand
FGF-23	Fibroblast growth factor - 23
G-6-P	Glucose-6-phosphate
GAD65	Glutamic acid decarboxylase 65
HbA1c	Glycated haemoglobin
HWE	Hardy-Weinberg equilibrium
HD	Hinge domain
HLA	Human leukocyte antigen
IA-2	Insulinoma-associated antigen-2
IAA	Insulin autoantibody
IDDM	Insulin dependent diabetes mellitus
lFNγ	Interferon gamma
INS	Insulin
IL	Interleukin
IS	Internal standard

LBD	Ligand binding domain
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT1	Nuclear factor activated T-cells 1
NFκB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
PTPN22	Protein tyrosine phosphate non-receptor type 22
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Reverse transcriptase
RXR	Retinoid X receptor
SANBS	South African National Blood Services
SNP	Single nucleotide polymorphism
SST	Serum separator tube
T1D	Type 1 diabetes
TBE	Tris borate EDTA
TCR	T cell receptor
TGF-β1	Transforming growth factor-β1
Th	T helper
TNFAIP3	Tumour necrosis factor alpha-induced protein 3
TNFα	Tumour necrosis factor alpha
UK	United Kingdom
USA	United States of America
UVB	Ultraviolet B rays
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D response elements
WHO	World Health Organisation
ZnT8	Zinc transporter-8
α	Alpha

β	Beta
Δ	Delta
f	Frequency
γ	Gamma
κ	Kappa
MØ	Macrophage
˚C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
bp	Base pair
kb	Kilo base
cm	Centimetre
kg/m^2	Kilogram per metre squared
ml	Millilitre
mM	Millimolar
mmol/L	Millimolar per litre
ng	Nanogram
nm	Nanometre
nmol/L	Nanomoles per litre
U	Units
U/µl	Units per microlitre
U/ml	Units per millilitre
g	Gravitational force

List of Figures

Figure 1.1: Schematic representation of the synthesis of vitamin D 2
Figure 1.2: Schematic representation of the structure of the VDR protein 3
Figure 1.3: Genetic mechanism of action of vitamin D 4
Figure 1.4: Stages in the development of T1D.....5
Figure 1.5: Loci associated with an increased risk of T1D (Zhang and Eisenbarth, 2010).....7
Figure 1.6: Immune pathogenesis of T1D 9
Figure 1.7: VDR signalling in T cells.....12
Figure 3.1: Box and whisker plot showing the vitamin D concentrations (nmol/L) in the total cohort, based on the season of recruitment.....35
Figure 3.2: Distribution of age at diagnosis in Indian and White T1D participants 39
Figure 3.3: Restriction enzyme digestion profiles for the *VDR* ((A) *Bsm*I, (B) *Fok*I, (C) *Apa*I and (D) *Taq*I) and vitamin D metabolising enzyme gene ((E) *CYP2R1* and (F) *CYP27B1*) polymorphisms run on a 2% agarose gel.....41
Figure 3.4: Chromatograms showing the portion of the *CYP2R1* gene containing the *CYP2R1* polymorphism, from individuals genotyped as (A) homozygous G, (B) homozygous A and (C) heterozygous GA for the *CYP2R1* polymorphism through PCR-RFLP. The box indicates the position of the polymorphism in the chromatogram. 42
Figure 7.1: PCR products *VDR* ((A) *Bsm*I, (B) *Fok*I, (C) *Apa*I/ *Taq*I and vitamin D metabolising enzyme gene((D) *CYP2R1* and (E) *CYP27B1*) run on a 1% agarose gel 106
Figure 7.2: Chromatograms showing the portion of the *VDR* gene containing the *Bsm*I polymorphism, from individuals genotyped as heterozygous GA for the *Bsm*I polymorphism through PCR-RFLP. 107
Figure 7.3: Chromatograms showing the portion of the *VDR* gene containing the *Apa*I polymorphism, from individuals genotyped as heterozygous CA (top) and homozygous AA (bottom) for the *Apa*I polymorphism through PCR-RFLP. 107
Figure 7.4: Chromatograms showing the portion of the *VDR* gene containing the *Taq*I polymorphism, from individuals genotyped as homozygous TT (top) and heterozygous GT (bottom) for the *Taq*I polymorphism through PCR-RFLP. 108

Figure 7.5: Amplification (A) and melt (B) curves for quantification of the *VDR* gene 109
Figure 7.6: Amplification (A) and melt (B) curves for quantification of the *β-actin* gene 109
Figure 7.7: Amplification products of the *VDR* and *β-actin* genes used for relative quantification run on a 1% gel showing no contamination but primer dimer amplification in the negative controls.....110

List of Tables

Table 2.1: Primer sequences for the amplification of the four polymorphisms within the VDR gene 21
Table 2.2: Annealing temperatures required for the amplification of the regions containing the VDR gene polymorphisms 22
Table 2.3: Restriction enzymes and buffers used for digestion of VDR PCR products 23
Table 2.4: Fragment sizes generated from PCR-RFLP for genotyping of the four VDR polymorphisms.....24
Table 2.5: Primer sequences for the amplification of the region of the vitamin D metabolising enzyme genes containing *CYP2R1* and *CYP27B1* polymorphisms.....25
Table 2.6: Reagents for amplification of *CYP2R1* and *CYP27B1* polymorphisms 25
Table 2.7: Fragment sizes generated from PCR-RFLP for genotyping of the vitamin D metabolising enzyme gene polymorphisms.....26
Table 2.8: Primer sequences for VDR and *β-actin* gene amplification.....29
Table 2.9: Reagents for amplification of the *VDR* and *β-actin* genes 29
Table 3.1: Vitamin D status in the Indian and White population 34
Table 3.2: Anthropometric and clinical characteristics of the Indian and White populations 37
Table 3.3: Comparison of anthropometric and clinical characteristics between Indian and White T1D patients.....38
Table 3.4: Comparison of clinicopathological variables and ZnT8 AAb status in Indian and White T1D patients.....40
Table 3.5: HWE results for the *VDR* and vitamin D metabolising enzyme gene polymorphisms in Indian control participants 43
Table 3.6: HWE results for the *VDR* and vitamin D metabolising enzyme gene polymorphisms in White control participants 44
Table 3.7: Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in our Indian population and populations from the 1000 Genomes project 46
Table 3.8: Comparison of allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms between our White population and populations from the 1000 Genomes project.....47

Table 3.9: Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the Indian population 49
Table 3.10: Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the White population 51
Table 3.11: Association between *VDR* and vitamin D metabolising enzyme gene polymorphisms and clinicopathological variables in Indian T1D participants 54
Table 3.12: Association between *VDR* and vitamin D metabolising enzyme gene polymorphisms and clinicopathological variables in White T1D participants 56
Table 3.13: Comparison of the number of *VDR* risk alleles between T1D patients and controls in the Indian and White population 59
Table 3.14: Comparison of the number of vitamin D metabolising enzyme gene risk alleles between T1D patients and controls in the Indian and White population.....59
Table 3.15: Clinicopathological variables and the number of *VDR* gene risk alleles in the Indian and White T1D patients 60
Table 3.16: Clinicopathological variables and the number of vitamin D metabolising gene risk alleles in the Indian and White T1D patients 62
Table 3.17: Variables affecting levels of vitamin D3 in the total population 63
Table 3.18: Variables affecting levels of total vitamin D in the total population 64
Table 3.19: Variables affecting levels of vitamin D in the White population.....64
Table 3.20: Comparison of *VDR* mRNA expression between White T1D patients and control participants 65

1 Literature Review

1.1 Vitamin D

Vitamin D is a steroid hormone which maintains the intracellular and extracellular homeostasis of calcium and is important for bone health (Holick, 2008). Vitamin D has two major forms, vitamin D2 (ergocalciferol) and D3 (cholecalciferol) (Holick *et al*., 2011). Vitamin D2 is found in plants and fortified foods and can therefore be obtained through the diet (Kanis, 1982). Similarly, vitamin D3 can be obtained from the diet (egg yolk, liver, fatty fish and fish liver oils) and supplements (Holick *et al*., 2011; Lehmann and Meurer, 2010). However, the majority of vitamin D3 is synthesised in the suprabasal and basal layers of the skin (Lehmann and Meurer, 2010).

Vitamin D3 is synthesised via ultraviolet radiation (280 - 320 nm) of 7-dehydrocholesterol (7-DHC). The synthesis of vitamin D3 depends on several factors such as (i) the proportion and concentration of 7-DHC in the skin (ii) Ultraviolet B (UVB) radiation wavelength and energy of photons (iii) time of day, latitude and season (iv) the amount of melanin in the skin (v) temperature and (vi) age (Lehmann and Meurer, 2010).

1.1.1 Metabolism of vitamin D

Vitamin D2 and D3 are inactive and require several hydroxylation steps to become biologically active (Kanis, 1982; Lehmann and Meurer, 2010). Vitamin D binds to the vitamin D binding protein (VDBP) in the blood and is transported to the liver. In the liver vitamin D is hydroxylated at carbon (C)-25 by cytochrome P450 25-hydroxylase (encoded by the *CYP2R1* gene) forming 25-hydroxyvitamin D3 ($25(OH)D_3$; Figure 1.1) (Christakos *et al*., 2012). In the proximal tubules of the kidneys, $25(OH)D_3$ is further hydroxylated by the 1*α*-hydroxylase enzyme (encoded by the *CYP27B1* gene) giving rise to the biologically active 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) (Busta *et al*., 2011). The activity of 1*α*-hydroxylase is regulated by parathyroid hormone (PTH), calcitonin and fibroblast growth factor 23 (FGF-23) (Lehmann and Meurer, 2010; Bikle, 2009). PTH and calcitonin stimulate *CYP27B1* expression while FGF-23 inhibits its expression (Bikle, 2009). Excess $1,25(OH)_2D_3$ induces *CYP24* gene expression

(Takeyama *et al*., 1999). The *CYP24* gene encodes the 24-hydroxylase enzyme which produces the biologically inactive metabolite 24,25 dihydroxyvitamin D (by initiating catabolic degradation through the hydroxylation of $25(OH)D_3$ or $1,25(OH)_2D_3$ at position C-24). This inactive metabolite is then excreted (White, 2011).

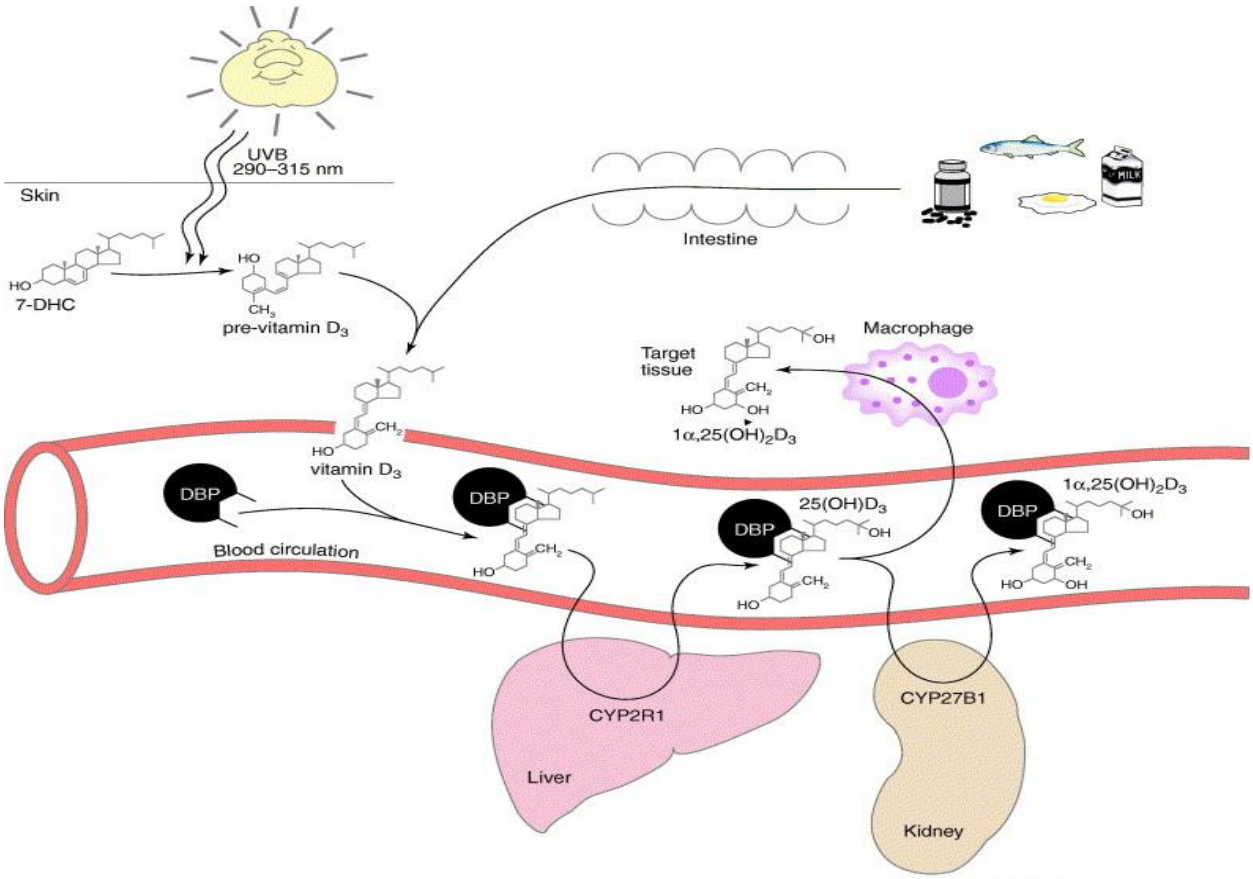

Figure 1.1: Schematic representation of the synthesis of vitamin D

Vitamin D is synthesised within the skin or obtained from the diet. In the skin, 7-DHC is converted to pre-vitamin D3 which spontaneously isomerises to vitamin D3. Vitamin D3 enters the circulation, where it is bound to VDBP allowing for transport of vitamin D within the body. In the liver, vitamin D3 is hydroxylated by 25-hydroxylase (encoded by *CYP2R1*) to $25(OH)D_3$. $25(OH)D_3$ is then transported to the kidneys for further hydroxylation by 1*α*-hydroxylase (encoded by *CYP27B1*) to give rise to the biologically active $1,25(OH)_2D_3$ (Mathieu and Badenhoop, 2005).

1.2 Vitamin D as an immune modulator

Vitamin D is a well-known regulator of bone metabolism and calcium homeostasis (Cantorna, 2010). In addition, vitamin D has a wide range of biological functions. These include inhibiting cellular proliferation, inducing terminal differentiation, inhibiting angiogenesis, stimulating insulin production, stimulating macrophage

cathelicidin (antimicrobial peptides) production and immune regulation (Harinarayan, 2014; Holick *et al*., 2011).

Vitamin D exerts its immunomodulatory effects by binding to the vitamin D receptor (VDR). The VDR consists of a highly conserved DNA binding domain (DBD), a C-terminal ligand binding domain (LBD) which binds $1,25(OH)_2D_3$ and a hinge domain (HD) which connects the two functional domains (Figure 1.2) (Matsuda and Kitagishi, 2013; Pike and Meyers, 2010; White, 2011). Most cells in the body express the VDR (Holick, 2003) including activated T and B lymphocytes and pancreatic β cells (Verstuyf *et al*., 2009).



Figure 1.2: Schematic representation of the structure of the VDR protein

The protein is comprised of a DNA binding domain (DBD), hinge domain (HD) and the C-terminal ligand binding domain (LBD) (Matsuda and Kitagishi, 2013).

The exact mechanism through which vitamin D/VDR signalling regulates the immune response is not fully understood. It has been proposed that VDBP transports $1,25(OH)_2D_3$ into the cells. Once in the cell, $1,25(OH)_2D_3$ dissociates from the VDBP and binds to the VDR in the cytoplasm (Figure 1.3). Binding of $1,25(OH)_2D_3$ results in a conformational change in the VDR which triggers binding to the retinoid X receptor (RXR). The $1,25(OH)_2D_3$-VDR-RXR complex enters the nucleus where it binds to specific sequences of DNA (termed vitamin D response elements (VDREs)) found within the promotor region of genes. The vitamin D complex either up or down regulates gene expression depending on the presence of inhibitory (co-repressors) or stimulatory (co-activators) proteins (Harinarayan, 2014). When the $1,25(OH)_2D_3$-VDR-RXR complex interacts with VDREs, co-repressors are released and nuclear receptor co-activator proteins are recruited which result in the induction of chromatin remodelling allowing for the binding of transcription factors and the initiation of transcription (Figure 1.3). Alternatively, when the $1,25(OH)_2D_3$-VDR-RXR complex

interacts with inhibitory VDREs, co-repressors are recruited resulting in inhibition of gene transcription (Baeke *et al*., 2010).

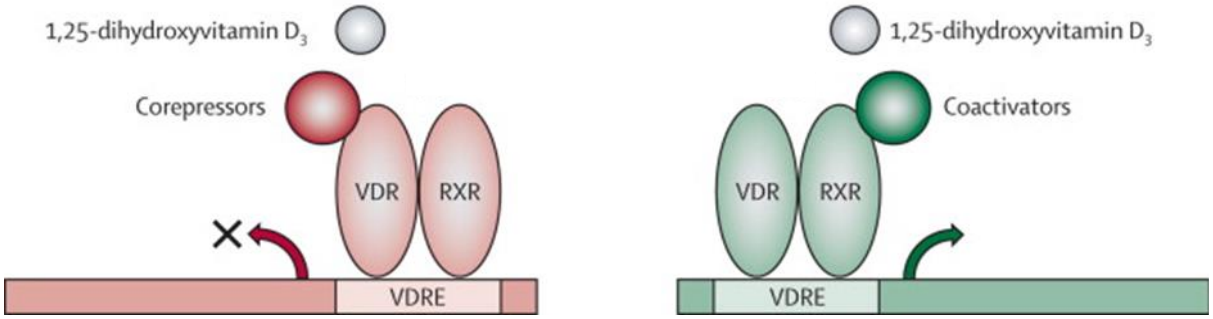

Figure 1.3: Genetic mechanism of action of vitamin D

$1,25(OH)_2D_3$ penetrates the plasma membrane and activates the VDR. The ligand bound receptor results in conformational changes and triggers binding to RXR. The complex binds to the VDRE and in the presence of co-activators or corepressors results in the upregulation or repression, respectively of the target gene (Janssens *et al*., 2013).

1.3 Vitamin D and its association with disease

Vitamin D deficiency has been associated with the development and progression of cardiovascular diseases and hypertension (Zhao *et al*., 2012), chronic kidney disease (Pilz *et al*., 2011), metabolic disease (Beydoun *et al*., 2010), psychiatric disorders (Humble *et al*., 2010) and cancer (Garland *et al*., 2005). In addition, vitamin D has been associated with autoimmune diseases such as multiple sclerosis (Royal III *et al*., 2009), rheumatoid arthritis (Merlino *et al*., 2004) and type 1 diabetes (T1D) (Hypponen *et al*., 2001).

1.4 Type 1 diabetes

T1D is an autoimmune disease that results in the destruction of β cells in the islets of Langerhans in the pancreas (Harris, 2005). Reduction in β cell population, persistent inflammation and lack of insulin decreases the uptake of glucose by body tissues (Wallberg and Cooke, 2013). The inability to maintain optimal blood glucose levels renders patients with T1D insulin dependent. Inadequate control of optimal blood glucose levels results in complications such as retinopathy, nephropathy, neuropathy,

and vascular complications (Boldison and Wong, 2016). Clinical symptoms occur when approximately 90% of the pancreatic β cells are destroyed (Mehra *et al.,* 2007). The main symptoms of the disease include polydipsia (excessive thirst), polyphagia (excessive hunger) and polyuria (excessive frequent urination) (Mehra *et al.,* 2007).

There are three stages for the development of T1D in genetically susceptible individuals (Figure 1.4). Firstly, an environmental trigger is necessary to elicit an immune response against β cells. Secondly, this response must be pro-inflammatory in nature to induce destruction of the β cells. Thirdly, there must be an ineffective regulatory mechanism against the immune response so that the response becomes chronic and leads to the destruction of the β cells (Wallberg and Cooke, 2013).

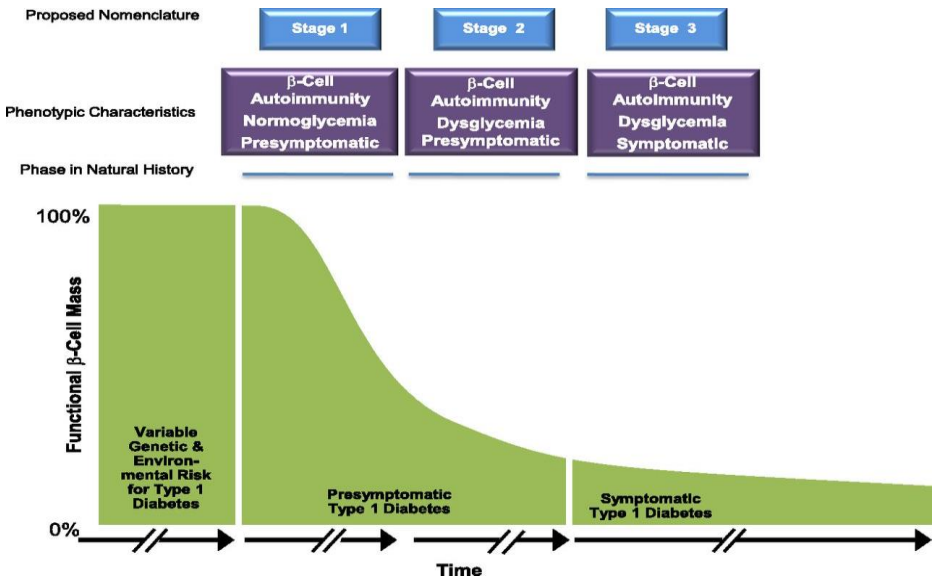

Figure 1.4: Stages in the development of T1D

β cell mass declines over time from stage 1 to 3. Stage 1 is characterised by the presence of two or more β cell specific autoantibodies in the presence of normoglycaemia. In stage 2, individuals develop dysglycaemia but are still presymptomatic. Stage 3 is the onset of symptomatic disease (Insel *et al*., 2015).

T1D is a multifactorial disease with both environmental (Knip *et al*., 2005) and genetic factors (Atkinson and Eisenbarth, 2001) having been implicated in its aetiology.

1.4.1 Environmental factors

Many studies implicate environmental factors in the pathogenesis of T1D. These environmental factors are said to play a crucial role in the initiation of an immune process which eventually leads to the destruction of pancreatic β cells. Environmental factors include exposure to certain viruses (e.g. enteroviruses, coxsackie viruses, rubella virus), environmental toxins, early exposure to a protein diet or cow's milk in infancy and vitamin D deficiency (Atkinson and Eisenbarth, 2001; Busta *et al*., 2011).

1.4.2 Genetic susceptibility

T1D is a polygenic disease and cannot be classified into a simple dominant, recessive or intermediate mode of inheritance. One of the earliest genes shown to be associated with the development of T1D are those located within the major histocompatibility complex (MHC) (Atkinson and Eisenbarth, 2001). In humans, the MHC is referred to as the human leukocyte antigen complex (HLA). The HLA region is located on chromosome 6p21.3, is about 4 000 kb in length and codes for over 200 genes.

HLA class II DR and DQ alleles confer approximately 40 - 50% of the genetic risk of developing T1D (Mehra *et al.,* 2007; Xie *et al*., 2014). The inheritance of HLA DR3 and DR4 alleles together with DQ2 and DQ8 alleles are strongly associated with susceptibility to T1D. These high risk alleles are expressed in more than 90% of T1D patients. The DR15 and DQ6 alleles are considered to be protective against T1D development (Szablewski, 2014).

Genome-wide association studies have identified over 40 loci that are associated with an increased risk for the development of T1D (Figure 1.5). These genes play a role in immunity (e.g. protein tyrosine phosphatase non-receptor type 22 (*PTPN22*)), insulin production and metabolism (e.g. insulin (*INS*)) and protection against β cell apoptosis (e.g. tumour necrosis factor alpha-induced protein 3 (TNFAIP3)) (Zhang and Eisenbarth, 2010). Of the non-HLA susceptibility genes, the *INS* and *PTPN22* genes confer the greatest risk of T1D development (Atkinson *et al*., 2014).

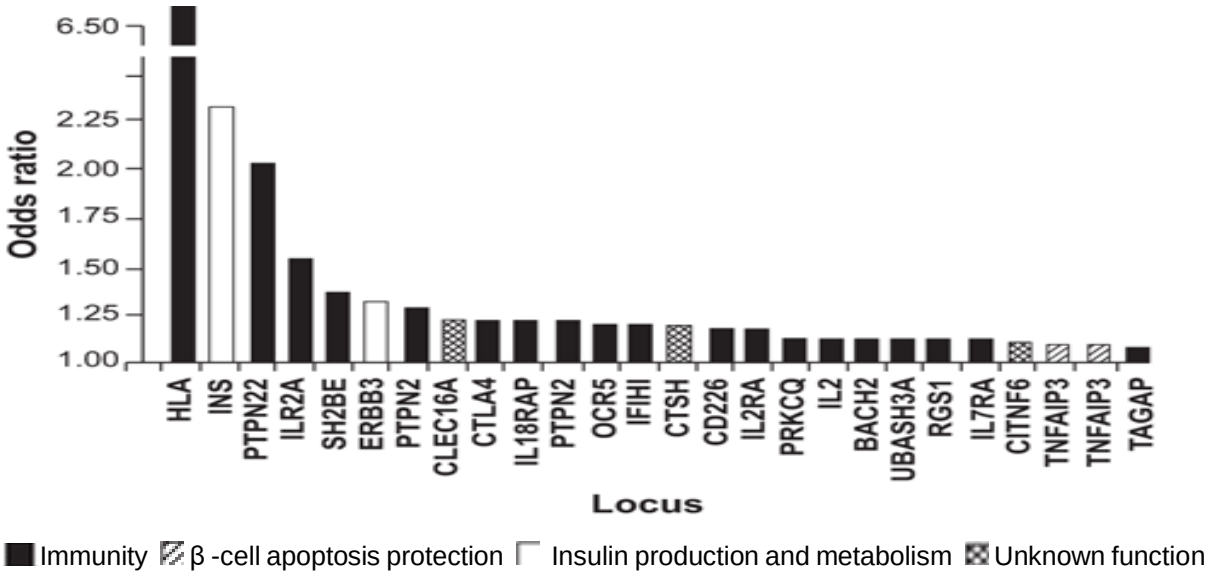

Figure 1.5: Loci associated with an increased risk of T1D (Zhang and Eisenbarth, 2010)

1.4.3 The pathogenesis of T1D

T1D is known to be a T-cell mediated disease (Devendra *et al*., 2004). T cells are divided into different subsets, namely T helper (Th)1, Th2, Th17 and regulatory T cells (Pietropaolo *et al*., 2008). Th1 cells mediate cellular immunity through secretion of pro-inflammatory cytokines (tumour necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin (IL)-2. In contrast, Th2 cells secrete anti-inflammatory cytokines (IL-4, IL-10 and transforming growth factor-β1 (TGF-β1)) and stimulate humoral immunity and the production of antibodies. Th1 and Th2 cells directly regulate each other's function via their specific cytokines (Almawi *et al*., 1999). A disruption of the equilibrium between the Th1/Th2 pathways is thought to play an important role in the pathogenesis of T1D (Busta *et al*., 2011). Th1 cells result in T1D through mediating the destruction of the β cells, therefore, a switch from the Th1 to Th2 pathway has a protective effect (Hill *et al*., 2003).

The exact mechanism involved in the initiation of T1D is still unknown. It is believed that following β cell injury, the antigen presenting cells (APC; macrophages or dendritic cells) present β cell-specific antigens via MHC class II molecules to naïve CD4+ Th0 cells (Figure 1.6) (Kukreja and Maclaren, 1999). This activates the APCs to secrete

IL-12 which results in the differentiation of the Th0 cells into Th1 cells. $CD4^+$ Th1 cells secrete IFNγ and IL-2. IFNγ stimulates resting macrophages to secrete cytokines such as IL-1β, TNFα and free radicals which are toxic to the pancreatic β cells. IL-2 induces the migration of β cell autoantigen specific $CD8^+$ cytotoxic T cells. These $CD8^+$ cytotoxic T cells damage β cells by releasing perforins and granzymes (serine proteases). After recognition of target cells, perforins and granzymes are released rapidly through exocytosis to induce granule-mediated cytotoxicity (Thomas *et al*., 2009; Padgett *et al*., 2013). Perforins form a pore on the β cell plasma membranes which allows the entry of cytotoxic granzymes. These granzymes then induce necrosis and apoptosis of β cells (Padgett *et al*., 2013).

β cell death may also be caused by Fas-mediated apoptosis. $CD8^+$ cytotoxic T cells are activated when the T cell receptor (TCR) binds to islet cell autoantigens presented by MHC class I molecules on the surface of β cells (Kukreja and Maclaren, 1999). Once activated, $CD8^+$ cytotoxic T cells induce β cell destruction through the binding of the Fas ligand (FasL) to its receptor on the β cell. This Th1 mediated destruction of the β cells results in T1D.

IL-4 mediated differentiation of CD4+ Th0 to Th2 cells results in β cell survival (Kukreja and MaClaren, 1999). This occurs through Th2 cell secretion of IL-4 and IL-10 which inhibits the proliferation of Th1 cells and the activation of macrophages by IFNγ (Liblau *et al*., 1995; Azar *et al*., 1999; Vaseghi and Jadali, 2016).

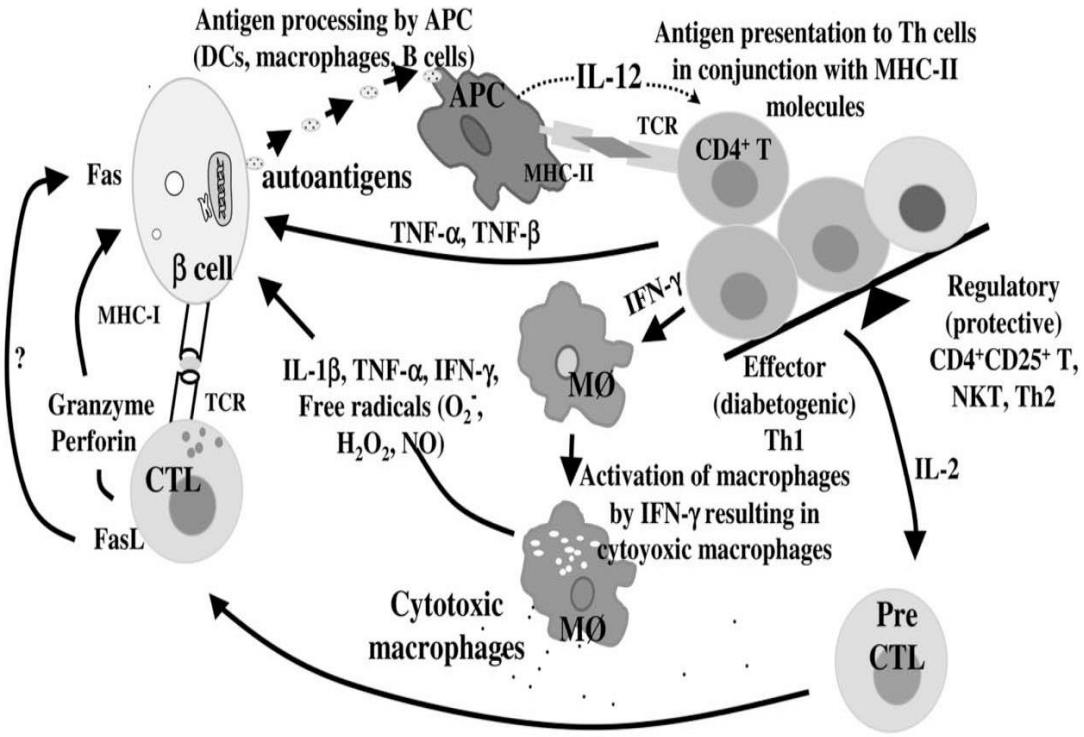

Figure 1.6: Immune pathogenesis of T1D

A shift from the anti-inflammatory Th2 to pro-inflammatory Th1 pathway results in activation of macrophages (MØ) and CD8+ cytotoxic T lymphocytes (CTL) which ultimately result in the destruction of the pancreatic β cells. β cells are destroyed via a number of mechanisms including Fas-mediated apoptosis and granzyme-mediated cytotoxicity damage by free radicles (Yoon and Jun, 2005).

1.4.4 Autoantibodies

Autoantibodies (AAbs; antibodies raised against self-antigens) are markers of T1D as they appear months to years before clinical onset of the disease (Hummel *et al*., 2004). The presence of multiple AAbs that persist over time is associated with a rapid clinical progression of the disease (Barker *et al.*, 2004*)*. The number, type and titre of the AAbs can predict the risk and rate of disease progression and the degree of severity of the clinical features (Schmidt *et al*., 2005). The Diabetes Prevention Trial Type-1, found that the five year T1D risk in participants with one AAb was 20 - 25%, 50 - 60% in those with two AAbs, 70% when three AAbs were present and nearly 80% when four AAbs were present (Atkinson, 2012). AAb positivity is influenced by the age of the patient, ethnicity, duration of disease and the screening method used (Wilmot-Roussel *et al.,* 2013).

Within T1D, AAbs target β cell antigens, including insulin (Palmer *et al*., 1983), the 65 kilodalton isoform of glutamic acid decarboxylase (GAD65; Baekkeskov *et al*., 1989), insulinoma-associated atigen-2 (IA-2; Passini *et al*., 1995) and zinc transporter 8 (ZnT8; Wenzlau *et al*., 2007).

Insulin AAbs are detected in about 40% of untreated children diagnosed with T1D (Morran *et al*., 2015). Once insulin therapy is initiated, the measurement of insulin AAbs is no longer appropriate due to AAbs directed to the exogenous insulin (Winter and Schatz, 2011). GAD65 AAbs are present in over 80% of newly diagnosed T1D patients and persist in the sera for many years following the diagnosis of T1D (Atkinson *et al*., 1994). The presence of GAD65 AAbs is a good predictor of future development of T1D in high risk populations. IA-2 AAbs are found in 60-70% of all patients and the frequency of IA-2 AAb positivity tends to decline with increasing disease duration (Raha *et al*., 2009; Hawa *et al*., 1997). ZnT8 AAbs are present in the serum of 60-80% of patients with new onset of T1D and disappear soon after clinical manifestation of the disease (Wenzlau *et al*., 2007).

1.4.5 The epidemiology of T1D

1.4.5.1 Age of onset

The onset of T1D generally occurs before the age of 30 years but peaks around puberty (10-14 years, Borchers *et al*., 2010). In the European population the age at diagnosis is reported to be between 0-14 years (Patterson *et al*., 2009) and 0-15 years in the Indian populations (Menon *et al*., 1990). This data is similar to that seen in the South African White population where the peak age at onset was 12-13 years (Kalk *et al*., 1993). Omar and Asmal (1984) showed that the mean age of onset in South African Indians was 17 years. In contrast, in Black South Africans with T1D, two peaks for the age of onset were observed; a major peak at 22-23 years of age with an earlier peak for age at onset at 14-17 years of age (Kalk *et al*., 1993).

1.4.5.2 Incidence of T1D

The incidence of T1D is increasing rapidly with the greatest increase seen in children less than five years of age (Weets *et al*., 2002). The incidence varies greatly depending on the geographical location and ethnicity of the population (Atkinson and Eisenbarth, 2001). The highest incidence of T1D is reported in Finland (>40/100 000/year) and Sardinia (37.8/100 000/year), while the lowest incidence rates are reported in Venezuela (0.1/100 000/year) and China (0.1–4.5/100 000/year) (Daneman, 2006; Borchers *et al*., 2009). In India, 13 years of collected data showed the incidence of T1D to be 3.8/100 000/year (Kumar *et al*., 2008).

1.4.5.3 Seasonal and geographical variation

Seasonal patterns of T1D onset have been observed with studies showing a greater number of new cases in the autumn and winter months (Dahlquist *et al*., 1994; Kahn *et al*., 2009). There is also wide variation in the geographical incidence of T1D with countries in the northern hemisphere having a higher incidence of the disease (north-south gradient i.e. lower UV radiation further from the equator and therefore a lack of sunlight) (Dahlquist *et al*., 1994; Kahn *et al*., 2009). Children born in spring are said to be at a higher risk of developing T1D due to low circulating levels of vitamin D in mothers and foetuses during winter pregnancies (Kahn *et al*., 2009). This suggests that there is an inverse relationship between levels of sunlight generated vitamin D and the incidence of T1D (Bailey *et al*., 2007). Low levels of vitamin D are thought to increase the risk of developing T1D via mediating the expression of pro-inflammatory cytokines. These effects of vitamin D are thought to be due to its role as an immune modulator.

1.5 Vitamin D as an immune modulator

Vitamin D is believed to protect against the development of T1D through pathways that regulate gene transcription (Figure 1.7). The VDR-RXR-vitamin D complex inhibits the transcription of the pro-inflammatory cytokine IL-2 through direct competition with the transcription factor nuclear factor activated T-cells 1 (NFAT1) for binding to the IL-2 gene promoter. In the absence of vitamin D, NFAT1, together with activator protein

1 (AP1), bind to the promoter resulting in increased expression of IL-2 (Kongsbak *et al*., 2013). In addition, the VDR-RXR-vitamin D complex can bind directly to the transcription factor nuclear factor kappa B (NFκB). This binding prevents NFκB binding to the IL-12 promoter which results in inhibition of IL-12 transcription. The VDR-RXR complex binds to the VDREs in the promotor region of the *IFNγ* gene resulting in decreased expression of IFNγ (Borges *et al*., 2010; Bikle, 2009). Vitamin D, therefore, downregulates the expression of the pro-inflammatory cytokines (IL-2, IL-12 and IFNγ) resulting in a switch from the Th1 to the Th2 pathway, suggesting a protective role for vitamin D in T1D (Jones *et al.,* 1998). Therefore, low levels of vitamin D can predispose individuals to T1D.

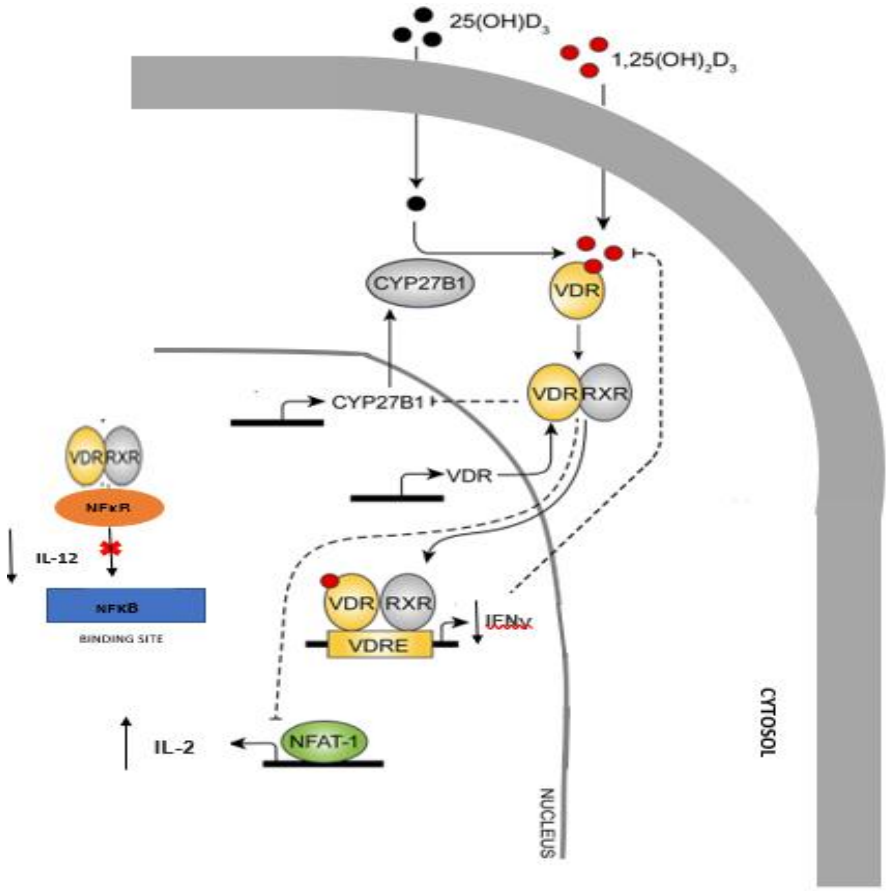

Figure 1.7: VDR signalling in T cells

Binding of $1,25(OH)_2D_3$ to the VDR results in a conformational change and subsequent heterodimerisation with RXR. This complex downregulates the expression of pro-inflammatory cytokines through binding to transcription factors such as NFκβ thereby preventing transcription of IL-12. In addition, this complex can bind to the promotor region of IL-2, which prevents the NFAT-1 transcription factor binding and thus inhibits IL-2 transcription. Finally, the complex is able to bind directly to VDREs and downregulate transcription of IFNγ (adapted from Kongsbak *et al*., 2013).

1.6 Vitamin D deficiency

There is a global pandemic of vitamin D deficiency (defined as a vitamin D concentration less than 24.96 nmol/L) or insufficiency (vitamin D concentration between 24.96-49.92 nmol/L) (Gallagher and Sai, 2010; Holick, 2007; Chen and Holick, 2008). The prevalence of vitamin D deficiency ranges from 30% to 93% depending on the populations studied (McKenna, 1992; Nesby-O'Dell S *et al*., 2002; Isaia *et al*., 2003). In the United States of America (USA) approximately 36% of healthy young adults (18 - 29 years of age) have been reported to have low vitamin D levels (<50 nmol/L; Tangpricha *et al*., 2002). Similarly, 42% of black women (15 - 49 years old; Nesby-O'Dell *et al*., 2002), 41% of outpatients (49 - 83 years old; Malabanan *et al*., 1998) and approximately 57% of general medicine patients in Massachusetts (Thomas *et al*., 1998) had low vitamin D levels.

Studies have shown that individuals who emigrate from Asia to countries of higher latitude fail to maintain optimal levels of vitamin D (serum vitamin D levels >50 nmol/L) irrespective of age and gender (Arneil, 1975; Dunnigan *et al*., 1962; Holmes *et al*., 1973). Studies from North India found that 96% of neonates (Sachan *et al*., 2005), 91% of healthy school girls (Puri *et al*., 2008), 78% of healthy hospital staff (Arya *et al*., 2004) and 84% of pregnant women (Sachan *et al*., 2005) had hypovitaminosis. In addition, studies conducted on South Indian populations showed levels of vitamin D deficiency ranged from 44% to 83% (Harinarayan, 2005; Harinarayan *et al*., 2007; Harinarayan *et al*., 2008). Interestingly, a study conducted on Bangladeshi women showed that levels of vitamin D in this population were not influenced by age, lifestyle or clothing choice (Islam *et al*., 2006).

In the South African context, vitamin D deficiency was found to be higher in Indian (24 – 67 %) as compared to Black (5 - 36.5 %) and White (21.5 %) participants (George *et al*., 2014; Naidoo *et al*., 2018). In contrast, a study conducted in Gauteng showed that mean plasma vitamin D levels in Black and White individuals were within the normal range (84.4 nmol/L and 92.4 nmol/L, respectively) (O' Neill *et al*., 2013).

1.6.1 Vitamin D deficiency and T1D

Epidemiological studies have shown an association between vitamin D deficiency and T1D (Staples *et al*., 2003; Hypponen *et al*., 2001). Children at the age of one year, who are vitamin D deficient, have a fourfold greater risk of developing T1D than those who are considered to be vitamin D sufficient (Hypponen *et al*., 2001). Studies investigating the effect of vitamin D in early childhood showed that children that received vitamin D supplements were less likely to develop T1D than those who did not receive supplements (The EURODIAB Substudy 2 Study Group, 1999; Hypponen *et al*., 2001; Zipitis and Akobeng, 2008). In addition, the intake of vitamin D rich foods, by mothers during pregnancy (Fronczak *et al.,* 2003) or children in the first year of life (Stene *et al*., 2003), is significantly associated with a reduced risk of T1D development. However, a large cohort in Finland showed no association between the intake of vitamin D and the risk of T1D in offspring with a high genetic risk of T1D development (Li *et al*., 2014).

Vitamin D deficiency at the cellular level is not only due to environmental and social factors but due to variations in the *VDR* and vitamin D metabolising enzyme genes (Lemos *et al*., 2008; Mohammadnejad *et al*., 2012; Abd-Allah *et al*., 2014; Ramos-Lopez *et al*., 2007; Hussein *et al*., 2012).

1.7 *VDR* polymorphisms and T1D

Four polymorphisms within the *VDR* gene (*Bsm*I (rs1544410; Morrison *et al*., 1992), *Fok*I (rs10735810; Gross *et al*., 1996), *Apa*I (rs7975232; Faraco *et al*., 1989) and *Taq*I (rs721236; Morrison *et al*., 1994) have been shown to be associated with T1D (McDermott *et al*., 1997). These polymorphisms are commonly referred to by the name of the restriction enzymes used to genotype participants by PCR-RFLP analysis (Ginter and Simko 2012). For the purpose of this dissertation, we will be referring to the polymorphisms by the restriction enzyme used for genotyping.

1.7.1 The *Bsm*I polymorphism

The *Bsm*I polymorphism results from a G>A transition and is found in intron 8 of the *VDR* gene (Morrison *et al*., 1992). Numerous studies have looked at the association of the *Bsm*I polymorphism and T1D; however, the results have not been consistent. Studies conducted on Egyptian, Indian Asian and Greek populations showed that the A allele had a significantly higher frequency in T1D patients than controls and that this allele conferred a greater risk of developing T1D (Abd-Allah *et al*., 2014; McDermott *et al*., 1997; Panierakis *et al*., 2009). In contrast, a study conducted by Pani *et al*. (2000) showed the A allele to be protective in a German population. Similarly, studies conducted in Taiwanese and Japanese populations found the A allele of the *VDR Bsm*I polymorphism at a lower frequency in T1D patients when compared to control participants (Chang *et al*., 2000; Motohashi *et al.,* 2003). Studies conducted on Chilean, Portuguese, German, Japanese and Finnish populations showed that there was no difference in allelic frequency distribution between T1D patients and controls (Angel *et al*., 2004; Lamos *et al*., 2008; Nejentsev *et al*., 2004; Yokota *et al.,* 2002; Turpeinen *et al*., 2003).

1.7.2 The *Fok*I polymorphism

The *Fok*I polymorphism is found in exon 2 of the *VDR* gene and results from a T>C transition. The C allele results in the generation of a second initiation codon, leading to a more active *VDR* protein that is three amino acids shorter than the wildtype protein (Gross *et al*., 1998). Studies conducted on a Japanese population showed that the C allele was associated with an increased susceptibility to T1D (Ban *et al*., 2001). This is in contrast to studies conducted on Egyptian, North Indian, Dalmatian and Greek populations which showed that the T allele was significantly increased in patients and carried a higher risk of developing T1D (Abd-Allah *et al*., 2014; Israni *et al*., 2009; Zemunik *et al*., 2005; Panierakis *et al*., 2009). However, studies conducted on Portuguese, Iranian and Finnish populations showed that there was no association between the *Fok*I polymorphism and T1D (Lamos *et al*., 2008; Mohammadnejad *et al*., 2012; Turpeinen *et al*., 2003).

1.7.3 The *Apa*I polymorphism

The *Apa*I polymorphism results from a C>A transversion and is found in intron 8 of the *VDR* gene (Faraco *et al*., 1989). Studies conducted on Taiwanese and Greek populations showed that the A allele was more frequent in T1D patients as compared to control participants and was associated with an increased risk of T1D (Chang *et al*., 2000; Panierakis *et al*., 2009). Conversely, a study by Pani *et al*. (2000) on a German population showed that the A allele was the protective allele. Furthermore, studies conducted on Chilean, Portuguese, German, Japanese and Finnish populations failed to show an association between this polymorphism and T1D (Angel *et al*., 2004; Lamos *et al*., 2008; Nejentsev *et al*., 2004; Turpeinen *et al*., 2003).

1.7.4 The *Taq*I polymorphism

The *Taq*I polymorphism results in a silent T>C transition and is found in exon 9 of the *VDR* gene (Morrison *et al*., 1994). In studies conducted on Indian, Iranian and Romanian populations, the C allele of the *Taq*I polymorphism was shown to increase the risk of T1D (McDermott *et al*., 1997; Mohammadnejad *et al*., 2012; Guja *et al*., 2002). In contrast, a study conducted on a German population showed that the T allele had a higher prevalence in T1D patients as compared to controls (Fassbender *et al*., 2002). Similarly, a study conducted on another German population found the C allele to be protective (Pani *et al*., 2000). However, studies conducted on Chilean children, Portuguese, and Finnish populations failed to show an association between this polymorphism and T1D (Angel *et al*., 2004; Lamos *et al*., 2008; Turpeinen *et al*., 2003).

In addition to the *VDR* polymorphisms, polymorphisms in the vitamin D metabolising enzyme genes (*CY27B1* and *CYP2R1*) have been associated with T1D (Rose *et al*., 2013).

1.8 Polymorphisms in the vitamin D metabolising enzyme genes and their association with T1D

The metabolising enzyme gene polymorphisms will be referred to by their coding gene name, *CYP2R1* and *CYP27B1*.

1.8.1 The *CYP2R1* polymorphism

The *CYP2R1* gene encodes the cytochrome P450 enzyme 25-hydroxylase which catalyses the hydroxylation of cholecalciferol to 25(OH)D_3 (Christakos *et al*., 2012). The gene is located on chromosome 11p15.2, has five exons and is approximately 15.5 kb long (Cheng *et al*., 2003). A transition mutation (G>A; rs10741657) at position 99 of this gene results in the substitution of the amino acid proline with leucine. This substitution results in the elimination of 25-hydroxylase enzyme activity (Cheng *et al*., 2004). A study conducted by Hussein *et al*. (2012) in Egyptian children showed that patients with the GG genotype had a 2.6 fold increased risk of developing T1D. In addition, a study conducted on German individuals showed that the G allele of the *CYP2R1* polymorphism is frequently passed from mothers to T1D affected children, thus indicating the association of this allele with susceptibility to T1D. (Ramos-Lopez *et al*., 2007). Furthermore, in both these studies, the G allele was associated with lower vitamin D levels in patients compared to control participants (Hussein *et al*., 2012; Ramos-Lopez *et al*., 2007). In a British cohort, the *CYP2R1* AA genotype was shown to be protective against T1D (Cooper *et al*., 2011).

1.8.2 The *CYP27B1* polymorphism

The *CYP27B1* gene (located on chromosome 12q13.1-q13.3) encodes the cytochrome P450 1α-hydroxylase enzyme which hydroxylates 25(OH)D_3 to 1, 25$(OH)_2D_3$ (Busta *et al*., 2011). A polymorphism (rs10877012) located in the promoter region (-1260) results in a G>T substitution (Bailey *et al*., 2007). This polymorphism has been associated with an increased risk of T1D development. A study conducted by Ramos-Lopez *et al*. (2004) in a German population showed that the G allele was found at a higher frequency in patients with T1D compared to control participants. In addition, the GG genotype has been shown to be associated with decreased *CYP27B1* mRNA expression levels in German patients when compared to control participants (Ramos-Lopez *et al*., 2007). Furthermore, a study conducted on Egyptian children showed that patients with the GG genotype had a 3.7 fold increased risk of T1D development compared to control participants (Hussein *et al*., 2012). The G allele leads to decreased expression of 1α-hydroxylase mRNA, hence reduced production of the enzyme, leading to lower levels of 1,25$(OH)_2D_3$ and an increased risk of T1D

development (Amit *et al*., 2014). However, a study conducted by Fichna *et al*. (2010) on a Polish population showed no difference in the distribution of allelic and genotypic frequencies in T1D patients and controls.

There is sparse data on the role that vitamin D plays in T1D in the South African Indian and White populations. Thus, we investigated the associations of serum levels of vitamin D, *VDR* gene polymorphisms, vitamin D metabolising enzyme gene polymorphisms and *VDR* mRNA expression levels, on the development of T1D in the South African Indian and White populations.

1.9 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 South African Indian and White populations. Therefore, the specific objectives of the study were:

- To measure and compare serum vitamin D concentrations in South African Indian and White T1D patients (cases) and non-diabetic participants (controls)
- To screen participants for four polymorphisms in the *VDR* gene (rs1544410 [*Bsm*I], rs7975232 [*Apa*I], rs2228570 [*Fok*I], rs731236 [*Taq*I]) and compare allelic and genotypic frequencies between cases and controls and relate these to serum vitamin D levels
- To determine the prevalence of polymorphisms in genes involved in the metabolic pathway of vitamin D (*CYP2R1* [rs10741657] and *CYP27B1* [rs10877012]) and compare allelic and genotypic frequencies between cases and controls and relate these to serum vitamin D levels
- To determine ZnT8 autoantibody positivity in cases and controls
- To compare ZnT8 autoantibody positivity in patients across the genotypes of all polymorphisms studied and relate to circulating vitamin D levels

2 Methods

2.1 Participant recruitment

Clinically diagnosed Indian (n=83) and White (n=106) South African T1D patients were recruited, through convenience sampling, from Helen Joseph Hospital, Charlotte Maxeke Johannesburg Academic Hospital, Inkosi Albert Luthuli Central Hospital and the Centres for Diabetes and Endocrinology (CDE) in Houghton and Lenasia. Non-diabetic Indian (n=88) and White (n=87) participants were recruited from South African National Blood Services (SANBS) blood drives and Wits Medical School. Participants with clinical evidence of chronic pancreatitis, pregnancy related diabetes or type 2 diabetes were excluded from the study.

All participants were required to provide informed consent and complete a questionnaire (Appendix 1) after which anthropometric measurements (weight, height, hip and waist circumference) were taken. In addition, three blood tubes (1 x ethylenediamine tetra-acetic acid (EDTA), 1 x heparin and 1 x serum separator tube (SST)) were collected from all participants. A sodium fluoride tube was collected from control participants to confirm normoglycaemia (non-fasting glucose levels ≤11.1 mmol/L).

Ethics clearance was obtained from the Wits Human Ethics research committee and from the SANBS ethics committee (clearance certificates M180536 and 2014/19, respectively; Appendix 2).

2.2 Sample processing

Blood samples were centrifuged for 10 minutes at 770 x *g* in a Beckman Coulter Allegra X-22R centrifuge (Beckman Coulter, California, USA) to obtain the desired fractions. The buffy coats were collected from the EDTA and heparinised tubes and stored for subsequent DNA extraction and isolation of peripheral blood mononuclear cells (PBMC), respectively. Serum from the SST tubes and plasma from EDTA was removed and aliquoted into 1.5 ml microcentrifuge tubes for vitamin D and ZnT8 AAb analysis, respectively. All the samples were stored at -80˚C until required. The sodium

fluoride tubes were sent to the National Health Laboratory Service's Chemical Pathology Diagnostic Laboratory for random blood glucose measurement.

2.3 DNA extraction

DNA was extracted from buffy coats, using the FlexiGene DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All centrifugation steps were performed in a Hettich Mikro 200 centrifuge (Labcare Ltd, Buckinghamshire, England). FG1 lysis buffer (500 µl) was pipetted into 1.5 ml centrifuge tubes. Buffy coat (200 µl) was added and the tubes mixed by inversion. The tubes were centrifuged at 10 000 x *g* for 20 seconds. The supernatant was discarded and the tubes were inverted onto clean paper towel for two minutes. A 10:1 mixture of FG2 buffer and protease (100 µl) was added and the tubes vortexed to completely homogenise the pellets. The tubes were then centrifuged briefly and incubated at 65°C for five minutes. Isopropanol (100 µl) was added and the tubes were mixed by inversion until the DNA precipitate was visible. The tubes were then centrifuged for three minutes at 10 000 x *g* and the supernatants discarded. Ethanol (70%; 150 µl) was added and the tubes were vortexed for five seconds followed by centrifugation for three minutes at 10 000 x *g*. The supernatants were discarded and the DNA pellets air-dried. FG3 buffer (200 µl) was added, and the DNA resuspended by incubating the tubes for one hour at 65°C in a water bath. The DNA was stored at -20°C until needed.

2.4 Genotyping participants for polymorphisms in the *VDR* gene

The genotypes for the four *VDR* polymorphisms (*Bsm*I (rs1544410), *Apa*I (rs7975232), *Fok*I (rs2228570), *Taq*I (rs731236)) were determined by polymerase chain reaction (PCR) amplification and restriction enzyme digestion of the PCR products.

2.4.1 Polymerase chain reaction of the *VDR* gene

Primer sequences for the amplification of the portions of the *VDR* gene containing the relevant polymorphisms were obtained from the literature (Israni *et al*., 2009). The respective primer sequences and resultant PCR product size are shown in Table 2.1.

Table 2.1: Primer sequences for the amplification of the four polymorphisms within the *VDR* gene

***VDR* SNP**	**Primer sequence**	**PCR product size (bp)**
*Bsm*I G>A	F: 5′-CAACCAAGACTACAAGTACCGCGTCAGTGA-3′ R: 5′-AACCAGCGGGAAGAGGTCAAGGG-3′	822
*Fok*I T>C	F: 5′-AGCTGGCCCTGGCACTGACTCTTGCTCT-3′ R: 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′	267
*Apa*I C>A	F: 5′-CAGAGCATGGACAGGGAGCAAG-3′ R: 5′-GCAACTCCTCATGGCTGAGGTCTCA-3′	745
*Taq*I T>C	F: 5′-CAGAGCATGGACAGGGAGCAAG-3′ R: 5′-GCAACTCCTCATGGCTGAGGTCTCA-3′	745

PCR reactions were set up in 0.2 ml microcentrifuge tubes. Each reaction consisted of 1x Kapa Taq Ready Mix (New England Biolabs, Massachusetts, USA), 0.39 µM of both the forward and reverse primers (Inqaba Biotechnical Industries, Pretoria, South Africa), approximately 50 ng template DNA and distilled water to bring the final volume to 18 µl. Each run included a non-template control (negative control) to ensure that there was no contamination. The PCR reactions were carried out under the following conditions:

Initial denaturation: 95˚C for three minutes

Denaturation: 95˚C for 30 seconds
Annealing: 61 - 64.8˚C (Table 2.2) for 30 seconds } 30 cycles
Extension: 72˚C for one minute

Final extension step: 72˚C for one minute

Table 2.2: Annealing temperatures required for the amplification of the regions containing the *VDR* gene polymorphisms

***VDR* SNP**	**Annealing temperature**
*Fok*I	61.0˚C
*Bsm*I	61.2˚C
*Apa*I/ *Taq*I	64.8˚C

Following amplification of the DNA, PCR products were visualised on a 1% agarose gel to verify successful amplification.

2.4.2 Agarose gel electrophoresis

A 1% agarose gel was made by mixing 0.5 g of agarose (Thermo Scientific, Massachusetts, USA) in 50 ml of 1x Tris-Borate-EDTA (TBE) buffer (Tris: Sigma-Aldrich, St. Louis, Missouri, USA; Boric acid: Sigma- Aldrich, St. Louis, Missouri, USA; EDTA: VWR International, Pennsylvania, USA). The agarose-TBE mixture was heated to dissolve the agarose and allowed to cool. Ethidium bromide (VWR International, Pennsylvania, USA; 15 µg) was added to the gel prior to pouring. Ethidium bromide intercalates into double stranded DNA allowing for visualisation of the DNA fragments under UV light. Once the gel had set, 2 µl of PCR product (mixed with 0.4 µl of 6x loading dye) was loaded on to the gel. The gel was run at 100 volts for 30 minutes and visualised using the ChemiDoc MP Imaging System (Biorad Laboratories (Pty) Ltd., Hercules, California, USA).

2.4.3 Restriction Fragment Length Polymorphism (RFLP) for genotyping participants for the *VDR* polymorphisms

Successfully amplified products were digested with the appropriate restriction enzymes (Table 2.3) for genotyping. Restriction enzyme (5 – 10 units (U)), 1x restriction enzyme buffer, and 5 µl of PCR product were added to a 0.2 ml microcentrifuge tube and made up to a final volume of 20 µl with distilled water. The reagents were mixed and incubated at 37˚C for two hours and the enzymes

deactivated at 65˚C for 20 minutes with the exception of the *Taq*I digest which was incubated at 65˚C for 2 hours followed by enzyme deactivation at 80˚C for 20 minutes.

Table 2.3: Restriction enzymes and buffers used for digestion of VDR PCR products

***VDR* SNP**	**Restriction enzyme**	**Restriction enzyme buffer**	**Manufacturer**
*Bsm*I	*Bsm*I (10 U/µl)	Buffer R	Thermo Scientific, Waltham, Massachusetts, USA
*Fok*I	*Fok*I (5 U/µl)	CutSmart	New England Biolabs, Ipswich, Massachusetts, USA
*Apa*I	*Apa*I (10 U/µl)	Buffer B	Thermo Scientific, Waltham, Massachusetts, USA
*Taq*I	*Taq*I (10 U/µl)	Buffer *Taq*I	Thermo Scientific, Waltham, Massachusetts, USA

The digested product (15 µl) was mixed with 2.5 µl of 6x loading buffer and run on a 2 % agarose gel at 100 volts for 45 minutes. The gels were visualised using the ChemiDoc MP Imaging System. Participants were genotyped based on the resultant fragment sizes (Table 2.4).

Table 2.4: Fragment sizes generated from PCR-RFLP for genotyping of the four *VDR* polymorphisms

SNP	Restriction enzyme	Genotype and respective fragment sizes
*Bsm*I G>A	*Bsm*I	GG: 651 and 171 bp GA: 822, 651 and 171 bp AA: 822 bp
*Fok*I T>C	*Fok*I	TT: 206 and 61 bp TC: 667, 206 and 61bp CC: 267 bp
*Apa*I C>A	*Apa*I	CC: 528 and 217 bp CA: 745, 528 and 217 bp AA: 745 bp
*Taq*I T>C	*Taq*I	TT: 494 and 251 bp TC: 494, 293, 251 and 201 bp CC: 293, 251 and 201 bp

2.5 Genotyping participants for polymorphisms in the vitamin D metabolising enzyme genes

The genotypes of the two vitamin D metabolising enzyme gene polymorphisms (*CYP2R1* [rs10741657] and *CYP27B1* [rs10877012]) were determined by PCR amplification and restriction enzyme digestion of the PCR products.

2.5.1 Polymerase Chain Reaction of the vitamin D metabolising gene polymorphisms

Primer sequences used for the amplification of the portions of the *CYP2R1* and *CYP27B1* genes containing the polymorphisms were obtained from Hussein *et al*. (2012) (Table 2.5).

Table 2.5: Primer sequences for the amplification of the region of the vitamin D metabolising enzyme genes containing *CYP2R1* and *CYP27B1* polymorphisms

Gene	SNP	Primer sequence
CYP2R1	G>A	F: 5′-GGGAAGAGCAATGACATGGA-3’ R: 5′-GCCCTGGAAGACTCATTTTG-3′
CYP27B1	G>T	F: 5’-GTGTTCCCTAAGTGTTGTCTC-3′ R: 5′-GCTGACTCGGTCTCCTCTG-3′

A 287 bp and 317 bp region of the *CYP2R1* and *CYP27B1* genes, respectively, were amplified in a final volume of 20 µl according to Table 2.6.

Table 2.6: Reagents for amplification of *CYP2R1* and *CYP27B1* polymorphisms

Reagent	Manufacturer	Final concentration
2 x Kapa Taq Ready Master Mix	New England Biolabs, Massachusetts, USA	1x
10 µM forward primer	Inqaba Biotechnical Industries, Pretoria, South Africa	0.39 µM
10 µM reverse primer	Inqaba Biotechnical Industries, Pretoria, South Africa	0.39 µM
Template DNA	-	~50 ng
Distilled water	-	Variable

Each run included a non-template control (negative control) to ensure that there was no contamination. The PCR reactions were carried out under the following conditions:

Initial denaturation: 95˚C for three minutes

Denaturation: 95˚C for 30 seconds
Annealing: 57˚C (*CYP2R1*) and 61˚C (*CYP27B1*) for 30 seconds } 30 cycles
Extension: 72˚C for one minute

Final extension step: 72˚C for one minute

2.5.2 Restriction Fragment Length Polymorphism for genotyping participants for the *CYP2R1* and *CYP27B1* polymorphisms

Successfully amplified *CYP2R1* and *CYP27B1* PCR products were digested with *Mn*lI (10 U/µl; buffer G) and *Tfi*I (10 U/µl; buffer O), respectively. Briefly, 5 U of restriction enzyme, 1x restriction enzyme buffer and 5 µl of PCR product were added to a 0.2 ml microcentrifuge tube and made up to a final volume of 20 µl with distilled water. The reagents were mixed together and incubated at 37˚C for two hours and the enzymes deactivated at 65˚C for 20 minutes. The digests were run on a 2 % agarose gel at 100 volts for 45 minutes and visualised using the ChemiDoc MP Imaging System. Genotypes were assigned based on the fragment sizes generated (Table 2.7).

Table 2.7: Fragment sizes generated from PCR-RFLP for genotyping of the vitamin D metabolising enzyme gene polymorphisms

SNP	**Restriction enzyme**	**Genotype and respective fragment sizes**
CYP2R1 G>A	*Mn*lI (Thermo Scientific, Waltham, Massachusetts, USA)	GG: 149, 108 and 29 bp GA: 257, 149, 108 and 29 bp AA: 257 and 29 bp
CYP27B1 G>T	*Tfi*I (Thermo Scientific, Waltham, Massachusetts, USA)	GG: 176 and 141 bp GT: 317, 176 and 141 bp TT: 317 bp

2.5.3 Sanger sequencing `

Three samples (representing the three possible genotypes) for each SNP studied were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing to confirm the correct amplification of the region of interest and the correct genotyping by PCR-RFLP. Sequences were analysed using Sequencher software (Gene Codes Corporation, Michigan, USA).

2.6 Gene expression studies for the *VDR* gene

Gene expression studies for the *VDR* gene were performed on RNA isolated from the PBMCs of a subset of randomly selected T1D patients (n = 7) and control participants (n = 9).

2.6.1 Isolation of peripheral blood mononuclear cells

Buffy coats collected from heparinised vacutainer tubes were used for PBMC isolation through density gradient centrifugation using Ficoll (GE Healthcare Biosciences, Uppsala, Sweden). The buffy coats were diluted with 1ml of 1 x PBS and overlaid onto 1 ml of Ficoll in a 15 ml conical tube, ensuring that no mixing occurred. The tubes were centrifuged (Beckman Coulter, California, USA) at 400 x *g* for 30 minutes at room temperature with the brake switched off. Ficoll centrifugation separates blood into red blood cells, granulocytes, PBMCs and plasma layers (GE Healthcare Biosciences, 2014). The layer containing the PBMCs was aspirated into a 15 ml conical tube and 1 x PBS added to bring the volume to 10 ml. The PBS and PBMCs were mixed gently and centrifuged at 300 x *g* for 10 minutes at room temperature and the supernatant discarded. The pellet containing PBMCs was washed by the addition of 10 ml 1 x PBS followed by a 10 minute centrifugation step at 300 x *g*. The supernatant was discarded and the wash step repeated. The pellet was then resuspended in 1 ml 1 x PBS and stored at -80˚C until needed.

2.6.2 RNA extraction

RNA was extracted from PBMCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PBMCs (350 µl) were aliquoted into 1.5 ml microcentrifuge tubes. Buffer RLT (350 µl) was added to disrupt the cells. The cells were homogenised by pipetting the lysate up and down several times followed by centrifugation for two minutes at full speed (14 000 x *g*) in a Hettich Mikro 200 centrifuge (Labcare Ltd, Buckinghamshire, England). Ethanol (70%; 350 µl) was added to the lysate and the samples were mixed by pipetting. Half of the sample volume (525 µl) was then transferred to RNeasy spin columns in 2 ml collecting tubes and centrifuged for 15 seconds at 8 000 x *g*. The flow-through was discarded. As the maximum volume the spin column can accommodate is 700 ul, this process was

repeated to allow for all RNA in the sample to adhere to the column. RW1 (700 µl) was added to the RNeasy column and centrifuged for 15 seconds at 8 000 x *g* and the flow-through discarded. Buffer RPE (500 µl) was added to the column and centrifuged for 15 seconds at 8 000 x *g* and the flow-through discarded. An additional 500 µl buffer RPE was added to the column and the tubes centrifuged for two minutes at 8 000 x *g*. The collecting tubes were discarded with the flow-through. The RNeasy columns were placed into new 2 ml collecting tubes and centrifuged for one minute at 8 000 x *g* to remove any residual buffer. The collection tubes were discarded with the flow-through. The RNeasy columns were then placed in a new 1.5 ml tube, RNase free water (50 µl) added and tubes allowed to stand for one minute. The tubes were centrifuged for one minute at 8 000 x *g* to elute the RNA.

2.6.3 DNase digestion

Contaminating genomic DNA was removed from the eluted RNA sample through DNase I digestion (Sigma- Aldrich, St. Louis, Missouri, USA). The extracted RNA (6 µl), 10x reaction buffer (1 µl), DNase I (10 U/µl; 1 µl) and RNase-free water (2 µl) were added into a 0.2 ml microcentrifuge tube and mixed gently. The reaction was incubated for 15 minutes at room temperature, following which stop solution (1 µl) was added and the mixture incubated for an additional 10 minutes at 70˚C. The microcentrifuge tubes were briefly placed on ice and the purified RNA samples were subsequently used for cDNA synthesis.

2.6.4 cDNA synthesis

cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA), according to the manufacturer's instructions. Total RNA (50 ng) was added to sterile nuclease-free tubes (on ice) followed by the addition of oligo$(dT)_{18}$ primers (1 µl) and made up to a volume of 12 µl with nuclease-free water. The tubes were gently mixed, briefly centrifuged and incubated for five minutes at 65˚C before being returned to the ice. Reaction buffer (5x; 4 µl) was added to the RNA-primer mix followed by 1 U RiboLock RNase Inhibitor (20 U/µl), 10 mM dNTP mix (2 µl) and 10 U RevertAid M-MuLV Reverse Transcriptase (RT; 200 U/µl) to a final volume of 20 µl. A reverse transcriptase (RT) negative control

was included to control for genomic DNA contamination from the RNA extraction. The RT negative control has template in the reaction but no RT. The tubes were gently mixed, briefly centrifuged and incubated for an hour at 65˚C. The RT was deactivated at 75˚C for five minutes. The cDNA was stored at -70˚C until needed.

2.6.5 Quantitative real time PCR

Samples were run in duplicate with primers against *β-actin* (reference gene; designed by TIB MOLBIOL, Berlin, Germany) and the *VDR* gene (Israni *et al*., 2009) (Table 2.8). The *β-actin* and *VDR* gene were amplified in separate reactions using the same volume of cDNA from the same cDNA preparation to allow for relative quantification. PCR reactions were set up in 0.1 ml tubes and were amplified in a final volume of 25 µl according to Table 2.9. Each run included a non-template and RT negative control to ensure that there was no contamination.

Table 2.8: Primer sequences for *VDR* and *β-actin* gene amplification

Gene	**Primer sequence**	**PCR product size (bp)**
VDR	F: 5′-AGCTGGCCCTGGCACTGACTCTTGCTCT-3′ R: 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′	267
β-actin	F: 5'-CATGTACGTTGCTATCCAGGC-3' R: 5'-CTCCTTAATGTCACGCACGAT-3'	250

Table 2.9: Reagents for amplification of the *VDR* and *β-actin* genes

Reagent	**Manufacturer**	**Final concentration**
2 x Luna universal SYBR green qPCR Master Mix	New England Biolabs, Massachusetts, USA	1x
10 µM *VDR* or *β-actin* forward primer	Inqaba Biotechnical Industries, Pretoria, South Africa	0.16 µM
10 µM *VDR* or *β-actin* reverse primer	Inqaba Biotechnical Industries, Pretoria, South Africa	0.16 µM
Template cDNA	-	~ 50 ng
Distilled water	-	Variable

The quantitative real time PCR reactions were carried out under the following conditions:

Initial denaturation: 95˚C for two minutes

Denaturation: 95˚C for 30 seconds
Annealing: 61˚C for 30 seconds
Extension: 72˚C for one minute
} 40 cycles

The threshold line was set at a level such that the fluorescent intensity of the reaction was above the background levels. The cycle number at which the amplification curve rose above the threshold (Ct) was determined for both the *VDR* and *β-actin* PCR reactions for each participant. The relative amount of *VDR* mRNA in each sample was calculated as the ratio between the Ct of the *VDR* PCR and the *β-actin* PCR (endogenous control) using the ΔCt method.

2.7 Measurement of serum vitamin D

The production of $25(OH)D_3$ is not regulated, therefore, serum concentrations of this metabolite reflect its dietary absorption and cutaneous synthesis (Harinarayan and Joshi, 2009). $25(OH)D_3$ has a half-life of 2-3 weeks which is considerably longer than that of $1,25(OH)_2D_3$, which is approximately four hours. In addition, the circulating levels of $1,25(OH)_2D_3$ are a thousand fold less than that of $25(OH)D_3$ (Holick, 2009). Thus, the most reliable indicator of vitamin D sufficiency is the measurement of serum $25(OH)D_3$.

Vitamin D levels were, therefore, assessed by measuring vitamin D3 on the Shimadzu Nextera High Performance Liquid Chromatography (HPLC) instrument using the ClinRep HPLC Complete Kit (Recipe, Munich, Germany).

2.7.1 Extraction of vitamin D

Vitamin D was extracted using the ClinRep HPLC Complete Kit according to a modified version of the manufacturer's protocol. Briefly, precipitation reagent P (250 µl) was pipetted into microcentrifuge tubes together with 200 µl internal standard (IS) and 200 µl of either the serum sample, control or standard. For a three point standard curve, standard one was made with 50 µl serum calibrator and 150 µl water, standard two with 100 µl serum calibrator and 100 µl water and standard three with 200 µl serum calibrator only. The IS was added last and kept at -20˚C until needed. The tubes were each vortexed for 30 seconds and then centrifuged in a Hettich Mikro 200 centrifuge (Labcare Ltd, Buckinghamshire, England) for five minutes at 10 000 x *g*. The upper phase of the supernatant was collected and 50 µl was injected into an isocratic HPLC system.

Separation of vitamin D3, IS and vitamin D2 was performed on a reverse phase column with UV detection at a wavelength of 264 nm. Chromatograms were integrated and vitamin D quantitated using the heights of the peak and IS, as compared to the standard curve. The IS is similar to the analytes in terms of behaviour during sample preparation and chromatography. Any losses during the sample preparation are therefore taken into account by quantitation of the internal standard height. Extrapolation to 100% recovery allows one to establish the concentration of vitamin D in the sample.

Recovery rate = Area IS (sample)/Area IS (calibrator)
Concentration = Area (sample) x Concentration (calibrator)/Area (calibrator) x recovery rate.

Vitamin D status was assessed according to the criteria set out by the Department of Chemical Pathology diagnostic laboratory. Participants were classified as sufficient if they had vitamin D levels > 72.5 nmol/L; insufficient if they had vitamin D levels between 50.0 - 72.5 nmol/L and deficient if their levels were < 50.0 nmol/L.

2.8 Measurement of ZnT8 AAbs

ZnT8 AAb detection was performed using the EuroImmun Anti-ZnT8 ELISA kit (EuroImmun, Lübeck, Germany), according the manufacturer's instruction. Calibrators, negative controls, positive controls and patient plasma samples (25 µl) were pipetted into individual microplate wells. The first well was left for the blank. The plate was covered, placed on a THERMOstar shaker (BGM labtech, Ortenberg, Germany) for five seconds at 500 rpm and incubated at 8˚C for 20 hours. The wells were emptied and washed three times with 1x wash buffer (350 µl). Excess liquid in the wells was discarded by tapping the plate face down on absorbent paper. Cold biotin-labelled ZnT8 (100 µl) was pipetted into each well with the exception of the blank well. The plate was covered and incubated at 8˚C for one hour. The plate was washed as previously described. Peroxidase-labelled avidin (enzyme conjugate; 100 µl) was pipetted into each well with the exception of the blank well. The plate was covered and incubated at room temperature for 20 minutes on a plate shaker at 500 rpm. The plate was washed as described above. Chromogen/substrate solution (100 µl) was pipetted into each well. The plate was incubated at room temperature for 20 minutes in the dark. The reaction was stopped with the addition of 100 µl stop solution to each well. The plate was shaken to ensure homogenous distribution of the solution and measured at a wavelength of 450 nm and 405 nm. All samples with a concentration greater than calibrator 3 (75 relative (R) U/ml) were read on the 405 nm standard curve and those with a concentration less than calibrator 3 were read from the 450 nm standard curve.

2.9 Measurement of serum glucose

The blood glucose levels were measured on the Roche Cobas® 6000 analyser series at the Charlotte Maxeke Johannesburg Academic Hospital, NHLS Chemical Pathology laboratory using the Glucose Hexokinase Generation 3 (GLUH_3) method. The principle of this method is described below.

Hexokinase catalyses the phosphorylation of glucose to glucose-6-phosphate (G-6-P) by adenosine triphosphate (ATP). In the presence of nicotinamide adenine dinucleotide phosphate (NADP), G-6-P is oxidised to gluconate-6-phosphate and

NADPH by G-6-P dehydrogenase. The resultant NADPH is directly proportional to the glucose concentration. Thus, photometric measurement of NADPH at 340/410 nm is used to determine the glucose concentration.

2.10 Statistical analysis

Statistical analysis was performed using Statistica software Version 13.5.0.17 (TIBCO software Inc., California, USA). Normally distributed data was represented as mean ± standard deviation. Data that was not normally distributed was log transformed before use and represented as median [upper quartile; lower quartile]. Categorical data were compared between cases and controls and across genotype groups using the Chi-squared $(\chi)^2$ test. Continuous variables between two groups were compared using the students t-test and for more than two groups an Analysis of variance (ANOVA) was used. Correlation between continuous variables was analysed using Pearson correlation. A stepwise backwards multiple regression analysis was used to isolate the principal determinants of serum vitamin D levels. Minor genotypes were combined with heterozygous genotypes for statistical analysis. The control population was used to calculate Hardy-Weinberg equilibrium (HWE) and for the comparison with polymorphism alleles from the 1000 Genome study populations. T1D participants were omitted from these analyses as they are expected to have higher frequencies of the risk alleles and thus may skew the data. Phi was used to represent the effect size. Statistical differences were considered significant if $p < 0.05$.

3 Results

3.1 Characteristics of the study population

A total of 364 participants were recruited for this study. The study population consisted of 171 Indian participants (83 T1D patients and 88 control participants) and 193 White participants (106 T1D patients and 87 control participants).

Vitamin D levels in the total cohort ranged from 2.9 to 228.1 nmol/L with 46.5% of participants having sufficient vitamin D levels (> 72.5 nmol/L), 28.5% having insufficient levels (50.0 - 72.5 nmol/L) and 25.0% of participants being vitamin D deficient (< 50.0 nmol/L). In the Indian population, the median vitamin D levels were 56.2 nmol/L, with 41.9% of participants being vitamin D deficient, 29.7% vitamin D insufficient and 28.4% having sufficient vitamin D levels. In the White population the median vitamin D levels were 78.1 nmol/L. The majority of the White participants (60.2%) had sufficient levels of vitamin D with 28.7% having insufficient levels and only 11.0% of participants being vitamin D deficient. There was no significant difference in the vitamin D status (deficient, insufficient or sufficient) between patients and controls in both the Indian and White population (p = 0.161 and p = 0.678, respectively; Table 3.1). The White population, as a whole, had higher vitamin D levels than the Indian population irrespective of whether they were patients or controls. This difference in vitamin D concentration is population specific. In addition, there was no difference in vitamin D levels based on the season during which participants were recruited (p = 0.075; Figure 3.1).

Table 3.1: Vitamin D status in the Indian and White population

Vitamin D status	**Indian**			**White**		
	T1D patients (%)	**Control participants (%)**	**p value**	**T1D patients (%)**	**Controls participants (%)**	**p value**
Deficient	49.4	34.6	0.161	12.9	8.8	0.678
Insufficient	27.3	32.1		26.7	27.5	
Sufficient	23.4	33.3		60.4	63.8	

*p value is significant at $p < 0.05$

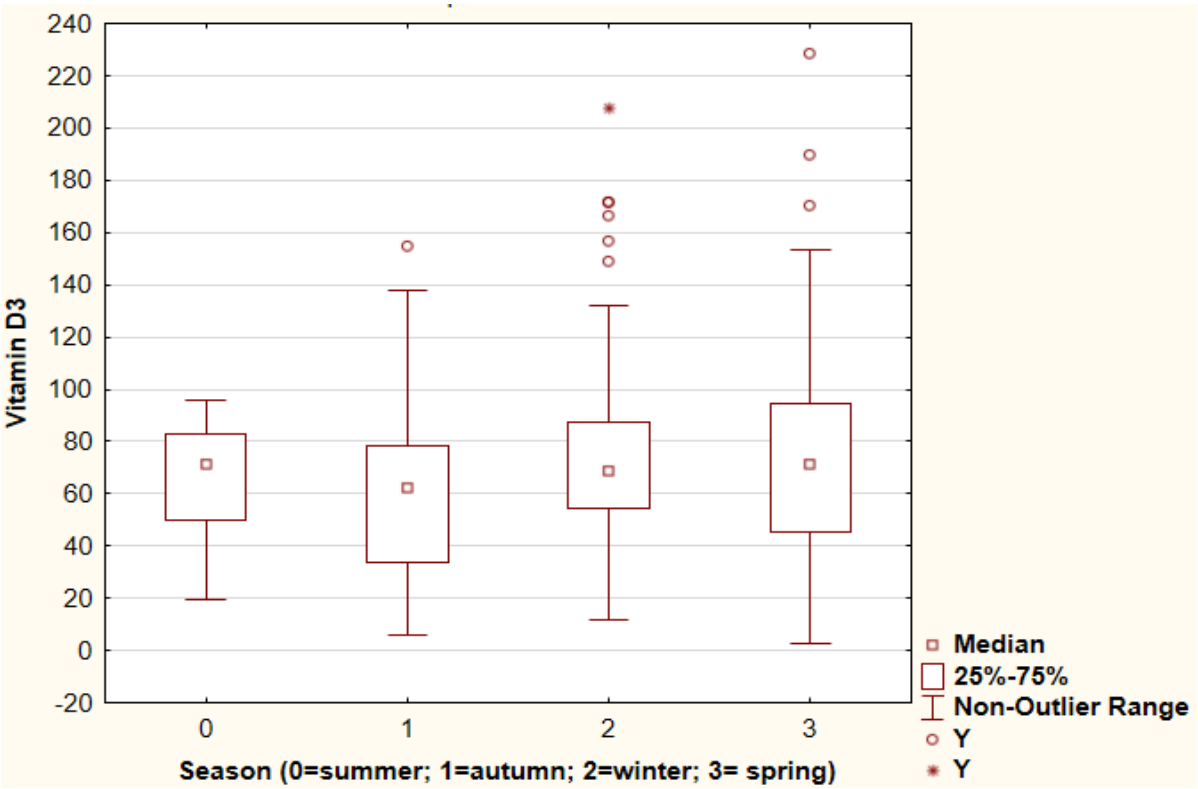

Figure 3.1: Box and whisker plot showing the vitamin D concentrations (nmol/L) in the total cohort, based on the season of recruitment

3.2 Anthropometric and clinical characteristics of participants in the Indian and White populations

The anthropometric and clinical characteristics of the Indian and White T1D patient and control participants are summarised in Table 3.2. Indian control participants were significantly older than Indian patients (34.1 vs. 25.1 years; p = 0.001), whereas White control participants were significantly younger than White patients (26.3 vs. 42.3 years; p <0.001). There were significantly fewer females in the Indian control group than in the patient group (36.6 vs. 52.4%; p = 0.041). In contrast, the White control group had significantly more female participants than the patient group (62.1 vs. 56.6%; p = 0.042). In addition, the White control group had a lower BMI (24.2 vs. 25.6 kg/m^2; p = 0.046) and smaller waist circumference (79.4 vs. 84.0 cm; p = 0.043) than the White T1D participants.

Glucose levels were significantly lower in both Indian and White control participants compared to their respective patient group (6.5 vs. 8.8 nmol/L; $p < 0.001$ and 5.4 vs. 7.0 nmol/L; $p < 0.001$, respectively).

In the Indian population the median age at which T1D was diagnosed was 13.0 years, with a mean duration of 11.7 years. In the White T1D patient group the median age at diagnosis was 20.0 years with a mean duration of 21.8 years. Approximately a fifth of the Indian patients (21.3%) and a quarter (23.6%) of White patients were ZnT8 AAb positive.

Table 3.2: Anthropometric and clinical characteristics of the Indian and White populations

Variables	Indian population			White population		
	T1D patients n = 83	Controls n = 88	p value	T1D patients n = 106	Controls n = 87	p value
Age (years)	25.1 ± 11.8	34.1 ± 16.1	<0.001*	42.3 ± 13.7	26.3 ± 9.7	<0.001*
Age at diagnosis (years)	13.0 [8.0; 17.0]	-	-	20.0 [11.0; 19.0]	-	-
Duration of disease (years)	11.7 ± 8.8	-	-	21.8 ± 12.6	-	-
Gender (% male)	47.6	63.4	0.041*	43.4	37.9	0.042*
Glucose (mmol/L)	8.8 [6.9; 11.8]	6.5 [5.2; 8.1]	<0.001*	7.0 [5.8;11.0]	5.4 [4.8; 6.5]	<0.001*
HbA1c (%)	8.8 [7.7; 10.0]	-	-	7.9 [7.1; 8.7]	-	-
BMI (kg/m^2)	24.2 ± 6.8	25.1 ± 5.0	0.342	25.6 ± 4.0	24.2 ± 5.6	0.046*
Waist circumference (cm)	85.3 [76.5, 96.0]	82.3 [75.5; 91.5]	0.111	84.0 [76.1; 93.0]	79.4 [71.6; 87.0]	0.043*
Vitamin D_3 (nmol/L)	49.6 [30.6; 70.2]	57.6 [34.6; 79.7]	0.271	74..8 [61.4; 95.5]	79.2 [59.8; 100.7]	0.235
Total vitamin D (nmol/L)	54.5 [32.1; 71.2]	59.2 [46.9; 76.4]	0.133	77.4 [64.5; 97.0]	79.9 [59.7; 100.3]	0.728
ZnT8 AAb positivity (%)	21.3	0.0	<0.001*	23.6	0.0	<0.001*

*p value is significant at $p < 0.05$; results expressed as mean ± standard deviation or median [lower quartile; upper quartile]; HbA1c = glycated haemoglobin, BMI = body mass index, ZnT8 AAb = zinc transporter 8 autoantibodies

3.2.1 Comparison of anthropometric and clinical characteristics between Indian and White T1D patients

Indian T1D participants were significantly younger (25.1 vs. 42.3 years; p <0.01), with a younger median age at diagnosis (13.0 vs. 20.0 years; p <0.001) and shorter disease duration (11.7 vs. 21.8 years; p <0.001) when compared to White T1D patients (Table 3.3). HbA1c levels were significantly higher in Indian compared to White T1D patients (8.8 [7.7; 10.0] vs. 7.9 [7.1; 8.7]; p <0.001). In addition, vitamin D levels were significantly lower in Indian T1D patients compared to White patients (vitamin D3: 49.6 [30.6; 70.2] vs. 74.8 [61.4; 95.5]; p <0.001 and total vitamin D: 54.5 [32.1; 71.2] vs. 77.4 [64.5; 97.0]; p <0.001).

Table 3.3: Comparison of anthropometric and clinical characteristics between Indian and White T1D patients

Variables	T1D Indian patients n = 84	T1D White patients n = 106	p value
Age (years)	25.1 ± 11.8	42.3 ± 13.7	<0.001*
Age at diagnosis (years)	13.0 [8.0; 17.0]	20.0 [11.0; 19.0]	<0.001*
Duration of disease (years)	11.7 ± 8.8	21.8 ± 12.57	<0.001*
Gender (% male)	47.6	43.4	0.570
Glucose (mmol/L)	8.8 [6.9; 11.8]	7.0 [5.8; 11.0]	0.088
HbA1c (%)	8.8 [7.7; 10.0]	7.9 [7.1; 8.7]	<0.001*
BMI (kg/m^2)	24.2 ± 6.8	25.6 ± 3.96	0.099
Waist circumference (cm)	85.3 [76.5, 96.0]	84.0 [76.1; 93.0]	0.066
Vitamin D_3 (nmol/L)	49.6 [30.6; 70.2]	74.8 [61.4; 95.5]	<0.001*
Total vitamin D (nmol/L)	54.5 [32.1; 71.2]	77.4 [64.5; 97.0]	<0.001*
ZnT8 AAb positivity (%, (n))	21.3 (17)	23.6 (25)	0.706

*p value is significant at $p < 0.05$; results expressed as mean ± standard deviation or median [lower quartile; upper quartile]; HbA1c = glycated haemoglobin, BMI = body mass index, ZnT8 AAb = zinc transporter 8 autoantibodies

3.2.2 Distribution of age at diagnosis of T1D participants

The majority (53%) of Indian patients were diagnosed between 6 and 15 years of age while two peaks for the age at diagnosis were seen in the White T1D patients (Figure 3.2). The first peak in the White T1D patients occurred at 11-15 years of age (19%) with the second peak occurring at 26-30 years of age (17%).

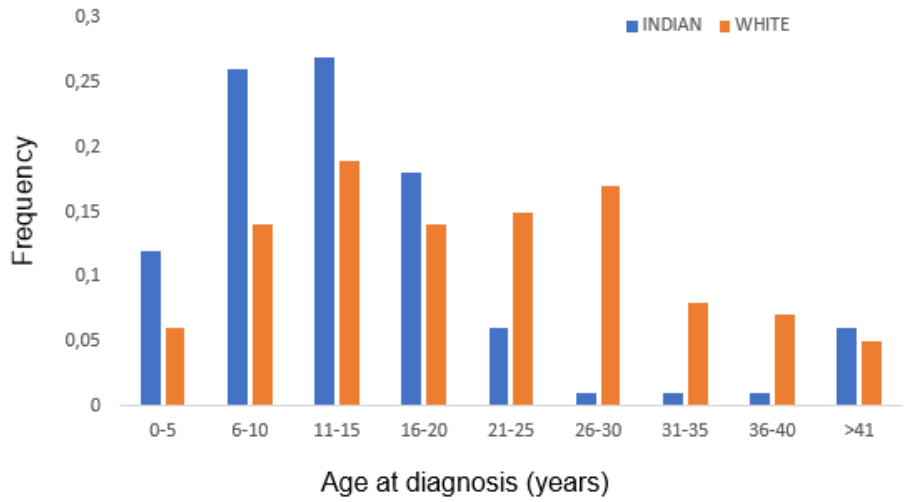

Figure 3.2: Distribution of age at diagnosis in Indian and White T1D participants

3.2.3 ZnT8 autoantibody status and clinicopathological variables in T1D patients

There was no statistically significant difference in age at diagnosis between individuals who were ZnT8 AAb positive and those who were ZnT8 AAb negative in either the Indian ($p = 0.130$) or White ($p = 0.266$) T1D patients (Table 3.4). When comparing disease duration between ZnT8 AAb positive and negative T1D patients, no difference was seen in the Indian cohort ($p = 0.526$). However, in the White T1D patients, the ZnT8 AAb positive participants had a significantly shorter disease duration when compared to the ZnT8 AAb negative participants ($p = 0.005$). No significant differences were observed for the remaining variables investigated.

Table 3.4: Comparison of clinicopathological variables and ZnT8 AAb status in Indian and White T1D patients

	AAb status in Indian T1D patients			**AAb status in White T1D patients**		
	Positive (n = 17)	**Negative (n = 63)**	**p value**	**Positive (n = 25)**	**Negative (n = 81)**	**p value**
Age at diagnosis (years)	8.5 [6.0; 12.5]	14.0 [10.0; 17.0]	0.130	20.0 [15.0; 25.0]	18.0 [11.0; 29.0]	0.266
Duration (years)	10.4 ± 7.3	12.0 ± 9.4	0.529	15.5 ± 9.7	23.6 ± 12.8	0.005*
BMI (kg/m^2)	21.1 ± 4.4	25.0 ± 7.1	0.044*	24.9 ± 3.6	25.8 ± 4.1	0.363
Glucose (mmol/L)	8.2 [5.6; 12.4]	8.9 [7.0; 11.8]	0.471	7.0 [4.7; 9.7]	7.5 [6.2; 11.0]	0.533
HbA1c (%)	9.6 [8.8; 10.5]	8.6 [7.6; 9.8]	0.198	7.8 [6.9; 8.3]	7.9 [7.1; 8.8]	0.445
Vitamin D3 (nmol/L)	39.9 [27.8; 65.6]	48.9 [3.3; 71.3]	0.860	75.5 [55.1; 93.2]	74.5 [61.4; 95.6]	0.783
Total vitamin D (nmol/L)	42.9 [27.8; 65.6]	52.8 [33.0; 75.9]	0.950	78.4 [64.5; 97.0]	76.6 [64.0; 97.2]	0.808

*p value is significant at $p < 0.05$; results expressed as mean ± standard deviation or median [lower quartile; upper quartile]; HbA1c = glycated haemoglobin, BMI = body mass index

3.3 Genotyping participants for the *VDR* and vitamin D metabolising enzyme gene polymorphisms

The DNA fragments containing the *VDR* and vitamin D metabolising enzyme gene polymorphisms were amplified using PCR (see Appendix 3 for gel images of PCR products). The PCR amplicons were digested with their respective restriction enzymes (see Table 2.4 and Table 2.7), run on a 2% agarose gel and participants genotyped based on the fragment patterns generated (Figure 3.3).

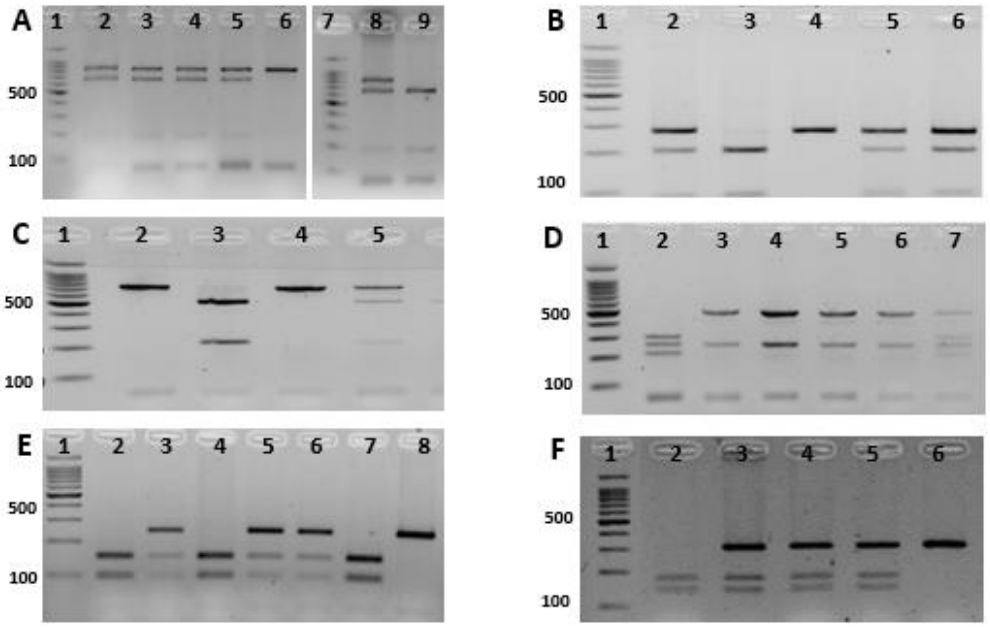

Figure 3.3: Restriction enzyme digestion profiles for the *VDR* ((A) *Bsm*I, (B) *Fok*I, (C) *Apa*I and (D) *Taq*I) and vitamin D metabolising enzyme gene ((E) *CYP2R1* and (F) *CYP27B1*) polymorphisms run on a 2% agarose gel

A: Lane 1 and 7: 100 bp DNA ladder, Lane 2-5 and 8: heterozygous participants (GA), Lane 6: participant homozygous for the A allele, Lane 9: participant homozygous for the G allele.
B: Lane 1: 100 bp DNA ladder, Lane 2 and 5: heterozygous participants (TC), Lane 3: participant homozygous for the T allele, Lane 4: participant homozygous for the C allele.
C: Lane 1: 100 bp DNA ladder, Lane 2 and 4: participants homozygous for the A allele, Lane 3: participant homozygous for the C allele, Lane 5: heterozygous participant (CA).
D: Lane 1: 100 bp DNA ladder, Lane 2: participant homozygous for the C allele, Lane 3-6: participants homozygous for the T allele, Lane 7: heterozygous participant (TC).
E: Lane 1: 100 bp DNA ladder, Lane 2, 4 and 7: participants homozygous for the G allele, Lane 3, 5 and 6: heterozygous participants (GA), Lane 8: participants homozygous for the A allele.
F: Lane 1: 100 bp DNA ladder, Lane 2: participant homozygous for the G allele, Lane 3-5: heterozygous participants (GT), Lane 6: participant homozygous for the T allele.

3.4 Sanger sequencing of *VDR* and vitamin D metabolising enzyme gene PCR products

Samples representing the different genotypes, determined through PCR-RFLP, for the *VDR* and vitamin D metabolising enzyme gene polymorphisms were sequenced. Sequencing was performed to confirm that participants were genotyped correctly by the PCR-RFLP assay. The sequencing results for the *CYP2R1* polymorphism are shown in Figure 3.4. Chromatograms for the remaining polymorphisms investigated can be found in Appendix 4.

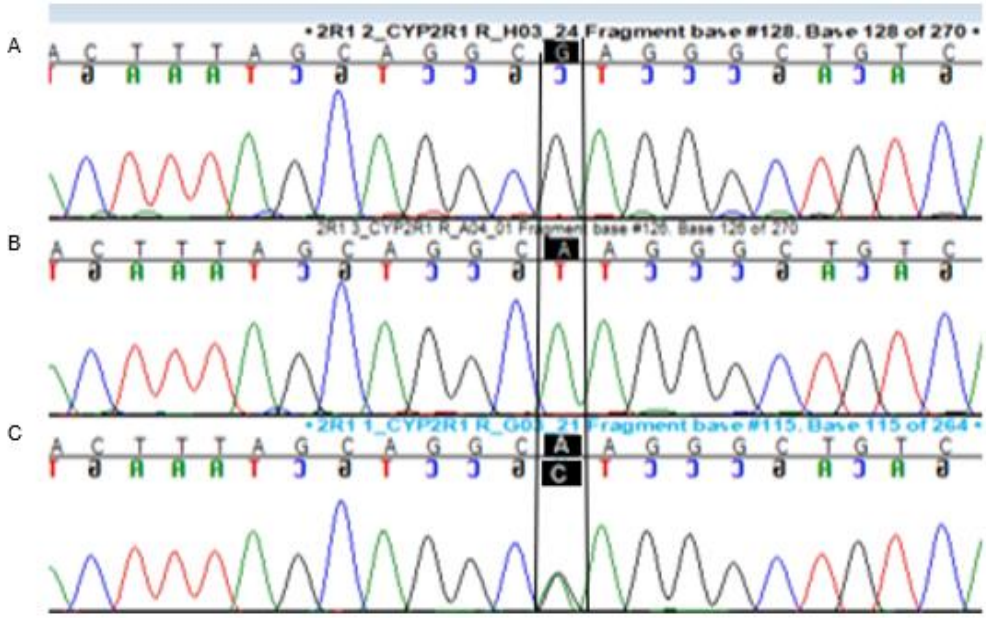

Figure 3.4: Chromatograms showing the portion of the *CYP2R1* gene containing the *CYP2R1* polymorphism, from individuals genotyped as (A) homozygous G, (B) homozygous A and (C) heterozygous GA for the *CYP2R1* polymorphism through PCR-RFLP. The box indicates the position of the polymorphism in the chromatogram.

3.5 Genotypic and allelic frequencies for the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the Indian and White control populations

The Indian and White control populations were determined to be in HWE for all polymorphisms investigated with the exception of *CYP27B1* which was not in HWE in either population (Table 3.5 and Table 3.6).

Table 3.5: HWE results for the *VDR* and vitamin D metabolising enzyme gene polymorphisms in Indian control participants

Genotype model	Participants (n)	χ^2	p value
***Bsm*I**			
GG	0.40 (34)	1.614	0.204
GA	0.41 (35)		
AA	0.19 (16)		
***Fok*I**			
TT	0.04 (3)	0.046	0.830
TC	0.29 (24)		
CC	0.67 (56)		
***Apa*I**			
CC	0.20 (17)	1.343	0.246
CA	0.42 (36)		
AA	0.38 (32)		
***Taq*I**			
TT	0.51 (42)	0.164	0.685
TC	0.40 (33)		
CC	0.10 (8)		
CYP2R1			
GG	0.52 (43)	0.097	0.756
GA	0.40 (33)		
AA	0.07 (6)		
CYP27B1			
GG	0.24 (19)	4.397	0.036*
GT	0.61 (49)		
TT	0.15 (12)		

*p value is significant at $p < 0.05$

Table 3.6: HWE results for the *VDR* and vitamin D metabolising enzyme gene polymorphisms in White control participants

Genotype model	Participants (n)	χ^2	p value
***Bsm*I**			
GG	0.34 (28)	0.024	0.878
GA	0.49 (41)		
AA	0.17 (14)		
***Fok*I**			
TT	0.12 (10)	1.107	0.293
TC	0.53 (43)		
CC	0.35 (28)		
***Apa*I**			
CC	0.22 (17)	1.080	0.299
CA	0.43 (33)		
AA	0.34 (26)		
***Taq*I**			
TT	0.38 (29)	0.021	0.884
TC	0.48 (37)		
CC	0.14 (11)		
CYP2R1			
GG	0.48 (39)	0.711	0.399
GA	0.40 (32)		
AA	0.12 (10)		
CYP27B1			
GG	0.21 (17)	6.036	0.014*
GT	0.63 (52)		
TT	0.16 (13)		

*p value is significant at $p < 0.05$

3.5.1 Comparison of allelic frequencies for *VDR* and vitamin D metabolising enzyme gene polymorphisms between the Indian and White South African control cohorts and populations in the 1000 genomes project

No significant differences in allelic frequencies were found between our South African Indian population and South Asian populations listed in the 1000 genomes project (Table 3.7). However, differences in allelic frequencies were observed for the four *VDR* polymorphisms ($p < 0.001$) and the *CYP2R1* ($p = 0.011$) polymorphism when comparing our Indian cohort to the 1000 genomes East Asian populations. In addition, our Indian population had a significantly lower *CYP27B1* G allele frequency than the 1000 genomes African (0.54 vs. 0.92; $p < 0.001$) and European (0.54 vs. 0.68; $p = 0.042$) populations.

No significant differences in allelic frequencies were found between our South African White and 1000 genomes European populations with the exception of the *CYP27B1* polymorphism (C allele frequency 0.52 vs. 0.68; $p = 0.021$) (Table 3.8). In contrast, the *Bsm*I G allele (0.58 vs. 0.27; $p = 0.026$), *Fok*I T allele (0.39 vs. 0.19; $p = 0.002$) and *CYP27B1* T allele (0.48 vs. 0.08; $p < 0.001$) frequencies were significantly higher in our White population compared to the 1000 genomes African populations. In addition, the *Bsm*I G allele (0.58 vs. 0.94; $p < 0.001$), *Apa*I C allele (0.44 vs. 0.71; $p < 0.001$), *Taq*I T allele (0.66 vs. 0.93; $p < 0.001$) and *CYP27B1* T allele (0.48 vs. 0.64; $p = 0.023$) frequencies were significantly higher in our White population compared to the 1000 Genomes East Asian populations.

Table 3.7: Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in our Indian population and populations from the 1000 Genomes project

SNP (allele *ƒ*)	Our study (Indian)	South Asian	p value	European	p value	African	p value	East Asian	p value
***Bsm*I (G allele)**	0.60	0.52	0.254	0.60	1.000	0.73	0.051	0.94	<0.001*
***Fok*I (T allele)**	0.18	0.26	0.172	0.38	0.002*	0.19	0.856	0.42	<0.001*
***Apa*I (C allele)**	0.42	0.41	0.886	0.45	0.669	0.36	0.384	0.71	<0.001*
***Taq*I (T allele)**	0.71	0.63	0.229	0.60	0.102	0.71	1	0.93	<0.001*
***CYP2R1* (G allele)**	0.73	0.64	0.171	0.62	0.097	0.78	0.411	0.68	0.438
***CYP27B1* (G allele)**	0.54	0.49	0.479	0.68	0.042*	0.92	<0.001*	0.36	0.011*

*p value is significant at *p* <0.05 when compared to the frequency of our Indian South African cohort, Genomes Project *et al*., 2015; *ƒ* = frequency

Table 3.8: Comparison of allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms between our White population and populations from the 1000 Genomes project

SNP (allele *ƒ*)	Our study (White)	European	p value	South Asian	p value	African	p value	East Asian	p value
***Bsm*I (G allele)**	0.58	0.60	0.774	0.52	0.394	0.73	0.026*	0.94	<0.001*
***Fok*I (T allele)**	0.39	0.38	0.884	0.26	0.050	0.19	0.002*	0.42	0.666
***Apa*I (C allele)**	0.44	0.45	0.887	0.41	0.668	0.36	0.248	0.71	<0.001*
***Taq*I (T allele)**	0.66	0.60	0.380	0.63	0.658	0.71	0.447	0.93	<0.001*
***CYP2R1* (G allele)**	0.68	0.62	0.374	0.64	0.551	0.78	0.111	0.68	1.000
***CYP27B1* (G allele)**	0.52	0.68	0.021*	0.49	0.671	0.92	<0.001*	0.36	0.023*

*p value is significant at p <0.05 when compared to the frequency of our White South African cohort, Genomes Project *et al*., 2015; *ƒ* = frequency

3.5.2 Genotypic and allelic associations of the *VDR* and vitamin D metabolising enzyme gene polymorphisms with T1D in the study cohort

Genotypic and allelic frequencies were compared between T1D and control participants for both the Indian (Table 3.9) and White (Table 3.10) populations.

Genotypic frequencies were significantly different between Indian T1D patients and control participants for the *Bsm*I, *Apa*I and *CYP2R1* polymorphisms (p = 0.023, 0.007 and 0.002, respectively). The T1D associated risk alleles for the Indian population were found to be the A allele for the *Bsm*I (0.53 vs. 0.39; p = 0.023), *Apa*I (0.72 vs. 0.59; p = 0.009) and *CYP2R1* (0.46 vs. 0.27; p <0.001) polymorphisms. The remainder of the polymorphisms investigated showed no association with T1D in the Indian population.

No statistically significant differences in the genotypic and allelic frequencies between the White T1D patients and control participants were found for any of the *VDR* polymorphisms studied, nor for the *CYP2R1* polymorphism. However, a significant difference in genotypic frequencies for the *CYP27B1* polymorphism was found between patients and controls (GG genotype: 0.49 vs. 0.21; p = 0.003). In addition, White patients were found to have a significantly higher frequency of the *CYP27B1* G allele compared to control participants (0.70 vs. 0.52; p = 0.001).

Table 3.9: Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the Indian population

SNP	**Frequency (n)**		χ^2	**p value (phi)**
	T1D patients	**Controls**		
***Bsm*I**				
Genotype model				
GG	0.20 (16)	0.40 (34)	7.553	0.023*
GA	0.56 (44)	0.41 (35)		(0.21)
AA	0.24 (19)	0.19 (16)		
Allele model				
G	0.47 (76)	0.61 (103)	5.151	0.023*
A	0.53 (82)	0.39 (67)		(0.13)
***Fok*I**				
Genotype model				
TT	0.13 (10)	0.04 (3)	4.418	0.110
TC	0.28 (22)	0.29 (24)		(0.16)
CC	0.60 (48)	0.67 (56)		
Allele model				
T	0.18 (42)	0.18 (30)	0.002	0.962
C	0.82 (188)	0.82 (136)		(0.00)
***Apa*I**				
Genotype model				
CC	0.04 (3)	0.20 (17)	10.046	0.007*
CA	0.47 (37)	0.42 (36)		(0.25)
AA	0.49 (38)	0.38 (32)		
Allele model				
C	0.28 (43)	0.41 (70)	6.656	0.009*
A	0.72 (113)	0.59 (100)		(0.14)

***Taq*I**				
Genotype model				
TT	0.39 (30)	0.51 (42)	2.985	0.225
TC	0.44 (34)	0.40 (33)		(0.14)
CC	17 (13)	0.10 (8)		
Allele model				
T	0.61 (94)	0.70 (117)	3.172	0.075
C	0.39 (60)	0.30 (49)		(0.10)
CYP2R1				
Genotype model				
GG	0.28 (22)	0.52 (43)	12.387	0.002*
GA	0.53 (42)	0.40 (33)		(0.28)
AA	0.20 (16)	0.07 (6)		
Allele model				
G	0.54 (86)	0.73 (119)	12.332	<0.001*
A	0.46 (74)	0.27 (45)		(0.20)
CYP27B1				
Genotype model				
GG	0.21 (16)	0.24 (19)	3.748	0.153
GT	0.51 (39)	0.61 (49)		(0.16)
TT	0.28 (21)	0.15 (12)		
Allele model				
G	0.47 (71)	0.52 (87)	1.832	0.176
T	0.53 (81)	0.46 (73)		(0.08)

*p value is significant at p <0.05

Table 3.10: Genotypic and allelic frequencies of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the White population

SNP	**Frequency (n)**		χ^2	**p value (phi)**
	T1D patients	**Controls**		
BsmI				
Genotype model				
GG	0.26 (29)	0.34 (28)	1.320	0.517
GA	0.60 (59)	0.49 (41)		(0.08)
AA	0.14 (14)	0.17 (14)		
Allele model				
G	0.57 (117)	0.58 (97)	0.044	0.841
A	0.43 (87)	0.42 (69)		(0.01)
***Fok*I**				
Genotype model				
TT	0.07 (7)	0.12 (10)	1.915	0.384
TC	0.60 (62)	0.53 (43)		(0.10)
CC	0.34 (35)	0.35 (28)		
Allele model				
T	0.35 (72)	0.39 (63)	0.501	0.479
C	0.65 (132)	0.61 (99)		(0.04)
ApaI				
Genotype model				
CC	0.22 (23)	0.22 (17)	1.398	0.550
CA	0.51 (52)	0.43 (33)		(0.09)
AA	0.26 (27)	0.34 (26)		
Allele model				
C	0.48 (98)	0.44 (67)	0.549	0.459
A	0.52 (106)	0.56 (85)		(0.04)

***Taq*I**				
Genotype model				
TT	0.38 (39)	0.38 (29)	0.018	0.990
TC	0.47 (48)	0.48 (37)		(0.01)
CC	0.15 (15)	0.14 (11)		
Allele model				
T	0.62 (126)	0.66 (115)	0.761	0.383
C	0.38 (78)	0.34 (59)		(0.04)
CYP2R1				
Genotype model				
GG	0.37 (39)	0.48 (39)	3.354	0.187
GA	0.42 (44)	0.40 (32)		(0.13)
AA	0.21 (22)	0.12 (10)		
Allele model				
G	0.58 (122)	0.68 (110)	3.747	0.053
A	0.42 (88)	32 (52)		(0.10)
CYP27B1				
Genotype model				
GG	0.49 (51)	0.21 (17)	15.850	0.003*
GT	0.40 (42)	0.63 (52)		(0.30)
TT	0.11 (11)	0.16 (13)		
Allele model				
G	0.70 (146)	0.52 (86)	11.412	0.001*
T	0.30 (64)	0.48 (78)		(0.18)

*p value is significant at p <0.05

3.6 Association of clinicopathological variables with *VDR* and vitamin D metabolising enzyme gene polymorphisms in the Indian and White T1D participants

We investigated whether the *VDR* and vitamin D metabolising enzyme gene polymorphisms studied were associated with any of the anthropometric and clinical characteristics in the Indian and White T1D patient groups (Table 3.11 and 3.12).

In the Indian patients, there was no statistically significant difference between the patient characteristics and genotypes, with the exception of the *Apa*I polymorphism. T1D patients with the combined *Apa*I CA and CC genotypes had an older age at diagnosis (15.0 [11.0; 19.0] vs. 10.5 [6.5; 15.5] years; p = 0.018) and higher glucose levels (10.2 [7.6; 13.4] mmol/L vs. 8.2 [5.8; 11.0]; p = 0.035) when compared to patients with the AA genotype.

No significant differences were found between any of the polymorphisms investigated and the clinicopathological variables in the White population.

Table 3.11: Association between *VDR* and vitamin D metabolising enzyme gene polymorphisms and clinicopathological variables in Indian T1D participants

Variables	*Fok*I			*Apa*I		
	CC	**TT + TC#**	**p value**	**AA**	**CC + CA#**	**p value**
Age at diagnosis (years)	13.5 [9.0; 17.0]	12.0 [8.0; 16.0]	0.810	10.5 [6.5; 15.5]	15.0 [11.0; 19.0]	0.018*
BMI (kg/m^2)	24.3 ± 7.1	24.5 ± 6.4	0.886	23.1 ± 5.4	25.5 ± 7.8	0.144
Glucose (mmol/L)	9.3 [6.9; 12.8]	8.6 [7.0; 10.5]	0.421	8.2 [5.8; 11.0]	10.2 [7.6; 13.4]	0.035*
HbA1c (%)	9.0 [7.7; 10.0]	8.8 [7.9; 10.5]	0.838	9.4 [8.1; 11.1]	8.6 [7.4; 9.6]	0.148
Vitamin D3 (nmol/L)	49.8 [30.3; 70.7]	49.7 [32.1; 70.4]	0.968	49.8 [32.1; 73.2]	49.7 [27.8; 65.0]	0.475
Total vitamin D (nmol/L)	75.8 [58.7; 106.4]	49.7 [32.1; 70.4]	0.884	52.1 [32.1; 83.0]	56.0 [30.1; 65.6]	0.586
ZnT8 AAb positivity (%, (n))	19.6 (9)	25.8 (8)	0.517	30.6 (11)	12.8 (5)	0.061

Variables	*Taq*I			*CYP2R1*		
	TT	**CC + TC#**	**p value**	**GG**	**AA + GA#**	**p value**
Age at diagnosis (years)	11.5 [9.0; 16.0]	14.0 [8.0; 18.0]	0.087	13.0 [9.0; 23.0]	12.0 [7.0; 17.0]	0.134
BMI (kg/m^2)	24.5 ± 7.1	24.3 ± 6.0	0.901	25.4 ± 7.8	24.0 ± 6.4	0.425
Glucose (mmol/L)	10.4 [7.0; 12..1]	8.3 [6.3; 11.0]	0.142	10.2 [8.2; 12.4]	6.9 [5.8; 8.7]	0.107
HbA1c (%)	9.6 [8.1; 11.7]	8.6 [7.8; 9.5]	0.256	9.6 [7.7; 11.5]	8.6 [7.7; 9.5]	0.070
Vitamin D3 (nmol/L)	48.0 [34.9; 61.4]	49.9 [28.3; 76.3]	0.914	38.1 [18.7; 71.3]	56.0 [34.4; 73.2]	0.241
Total vitamin D (nmol/L)	48.2 [35.2; 63.4]	57.8 [30.1; 82.9]	0.853	36.0 [16.6; 82.2]	56.2 [36.1; 71.2]	0.215
ZnT8 AAb positivity (%, (n))	21.4 (6)	19.6 (9)	0.867	20.0 (4)	22.8 (13)	0.795

Variables	*Bsm*I				*CYP27B1*			
	GG	GA	AA	p value	GG	GT	TT	p value
Age at diagnosis (years)	11.0 [8.0; 17.0]	14.5 [10.0; 18.0]	13.0 [8.0; 15.5]	0.500	11.0 [10.0; 16.5]	14.0 [8.0; 17.0]	13.5 [9.0; 19.0]	0.572
BMI (kg/m^2)	24.1 ± 6.1	24.4 ± 7.2	24.8 ± 6.8	0.961	22.5 ± 4.1	25.8 ± 8.0	24.4 ± 5.9	0.295
Glucose (mmol/L)	7.2 [6.5; 12.1]	9.7 [6.8; 12.8]	8.3 [7.0; 11.2]	0.829	11.0 [7.0; 14.6]	9.1 [7.0; 11.7]	9.8 [6.5; 10.8]	0.722
HbA1c (%)	8.6 [6.9; 9.7]	8.6 [7.6; 9.5]	9.5 [8.3; 11.7]	0.101	9.1 [8.6; 9.5]	8.6 [7.6; 10.0]	9.4 [7.9; 11.9]	0.477
Vitamin D3 (nmol/L)	39.5 [35.1; 61.4]	44.8 [30.6; 67.9]	55.9 [28.3; 92.7]	0.286	56.0 [34.6; 71.7]	49.8 [32.2; 72.6]	37.5 [27.8; 61.3]	0.493
Total vitamin D (nmol/L)	61.4 [49.5; 79.2]	56.0 [44.9; 81.3]	52.1 [32.1; 83.0]	0.274	56.0 [34.6; 71.9]	56.4 [34.6; 79.8]	37.5 [2 7.8; 61.4]	0.391
ZnT8 AAb positivity (%, (n))	20.0 (3)	18.6 (8)	26.3 (5)	0.790	31.3 (5)	18.4 (7)	20.0 (4)	0.567

*p value is significant at $p < 0.05$; results expressed as mean ± standard deviation or median [lower quartile; upper quartile]; HbA1c = glycated haemoglobin, BMI = body mass index, ZnT8 AAb = zinc transporter 8 autoantibodies, # homozygous T, C, C and A genotypes for the *Fok*I, *Apa*I, *Taq*I and *CYP2R1* polymorphisms, respectively, were combined due to small (n) number

Table 3.12: Association between *VDR* and vitamin D metabolising enzyme gene polymorphisms and clinicopathological variables in White T1D participants

Variables	*Fok*I			*Apa*I		
	CC	**TT + TC#**	**p value**	**AA**	**CC + CA#**	**p value**
Age at diagnosis (years)	17 [12.0; 25.0]	21.0 [12.0; 30.0]	0.426	21.5 [14.0; 31.0]	18.5 [11.0; 27.0]	0.306
BMI (kg/m^2)	25.0 ± 4.1	25.8 ± 3.9	0.394	25.4 ± 4.4	25.6 ± 3.8	0.776
Glucose (mmol/L)	7.5 [6.1; 10.0]	7.5 [5.7; 11.0]	0.711	8.7 [5.7; 11.3]	7.5 [5.8; 11.0]	0.850
HbA1c (%)	7.9 [7.2; 8.4]	7.9 [7.0; 8.8]	0.833	7.7 [7.0; 8.7]	7.9 [7.1; 8.7]	0.432
Vitamin D3 (nmol/L)	70.5 [55.1; 98.8]	74.8 [62.7; 88.9]	0.553	75.0 [63.0; 95.5]	74.0 [58.3; 87.7]	0.562
Total vitamin D (nmol/L)	75.8 [58.7; 106.4]	75.9 [64.5; 93.6]	0.789	86.5 [65.1; 106.4]	75.6 [63.3; 93.4]	0.299
ZnT8 AAb positivity (%, (n))	28.6 (10)	21.7 (15)	0.441	25.9 (7)	24.0 (18)	0.842

Variables	*Taq*I			*CYP2R1*		
	TT	**CC + TC#**	**p value**	**GG**	**AA + GA#**	**p value**
Age at diagnosis (years)	18.0 [11.0; 23.0]	20.5 [11.0; 29.0]	0.212	23.0 [11.0; 30.0]	18.0 [11.5; 25.0]	0.515
BMI (kg/m^2)	25.8 ± 4.2	25.5 ± 3.8	0.824	26.1 ± 4.1	25.3 ± 3.9	0.353
Glucose (mmol/L)	7.4 [5.8; 9.0]	8.3 [5.8; 12.0]	0.240	7.2 [5.4; 11.0]	7.5 [6.1; 11.0]	0.942
HbA1c (%)	7.9 [7.0; 8.7]	7.9 [7.2; 8.7]	0.673	7.7 [7.0; 8.6]	7.9 [7.2; 8.8]	0.628
Vitamin D3 (nmol/L)	74.8 [63.5; 87.68]	75.0 [57.9; 107.2]	0.678	69.6 [54.3; 83.5]	75.8 [63.0; 101.3]	0.077
Total vitamin D (nmol/L)	77.4 [69.6; 88.8]	77.7 [64.5; 107.2]	0.436	73.5 [56.7; 88.4]	78.3 [66.0; 103.6]	0.067
ZnT8 AAb positivity (%, (n))	20.5 (8)	27.0 (17)	0.460	23.1 (9)	24.2 (16)	0.892

Variables	*Bsm*I				*CYP27B1*			
	GG	**GA**	**AA**	**p value**	**GG**	**GT**	**TT**	**p value**
Age at diagnosis (years)	18.0 [11.0; 22.5]	21.0 [11.0; 29.0]	21.0 [16.0; 30.0]	0.408	20.0 [11.0; 29.0]	20.0 [13.0; 30.0]	25.0 [18.0; 30.0]	0.175
BMI (kg/m^2)	26.1 ± 4.0	25.6 ± 4.0	24.3 ± 4.4	0.411	25.7 ± 3.9	25.7 ± 4.0	24.2 ± 4.2	0.295
Glucose (mmol/L)	7.5 [6.3; 10.0]	7.0 [5.2; 10.9]	6.9 [5.2; 12.5]	0.939	7.5 [5.6; 11.0]	7.2 [5.8; 11.0]	9.0 [6.2; 12.0]	0.616
HbA1c (%)	8.2 [7.0; 8.8]	7.7 [7.1; 8.7]	7.8 [7.2; 8.7]	0.918	7.9 [7.1; 8.7]	8.0 [7.3; 8.8]	7.3 [7.0; 8.2]	0.408
Vitamin D3 (nmol/L)	74.9 [63.0; 88.4]	71.3 [57.8; 92.0]	88.8 [72.3; 106.4]	0.928	74.8 [61.4; 95.5]	73.7 [56.5; 103.8]	69.6 [58.7; 97.2]	0.985
Total vitamin D (nmol/L)	75.89 [69.2; 97.2]	74.5 [58.3; 93.4]	88.8 [72.3; 106.4]	0.605	76.7 [64.0; 97.0]	77.9 [63.6; 103.8]	69.6 [58.7; 97.2]	0.833
ZnT8 AAb positivity (%, (n))	20.7 (6)	25.4 (15)	21.4 (3)	0.868	29.4 (15)	21.4 (9)	9 (1)	0.315

*p value is significant at $p < 0.05$; results expressed as mean ± standard deviation or median [lower quartile; upper quartile]; HbA1c = glycated haemoglobin, BMI = body mass index, ZnT8 AAb = zinc transporter 8 autoantibodies # homozygous T, C, C and A genotypes for the *Fok*I, *Apa*I, *Taq*I and *CYP2R1* polymorphisms, respectively, were combined due to small (n) number

3.7 The association of increased numbers of *VDR* and vitamin D metabolising enzyme gene polymorphism risk alleles with T1D in Indian and White populations

Each individual can have a maximum of eight, and a minimum of zero, VDR risk alleles with respect to the four *VDR* polymorphisms. Similarly, individuals can have a maximum of four, or a minimum of zero, vitamin D metabolising gene risk alleles with respect to the *CYP2R1* and *CYP27B1* polymorphisms. For the purpose of this study, the *VDR* risk alleles were defined as *Bsm*I: A, *Fok*I: C, *Apa*I: A and *Taq*I: C. The vitamin D metabolising risk alleles were defined as *CYP2R1*: A and *CYP27B1*: G. The *Fok*I and *Taq*I risk alleles were based on data from the literature whereas the *Bsm*I, *Apa*I, *CYP2R1* and *CYP27B1* risk alleles were chosen based on the associations found within our study. The effect of increasing numbers of these T1D risk alleles on the development of T1D in the Indian and White populations was assessed (Table 3.13 and Table 3.14). In the Indian population, control participants had significantly fewer *VDR* and vitamin D metabolising enzyme gene risk alleles than Indian T1D patients ($p = 0.027$ and $p = 0.016$, respectively). There was no association with the number of risk alleles and the presence of T1D in the White population ($p = 0.872$ and $p = 0.412$).

3.7.1 Comparison of clinicopathological variables and the number of *VDR* gene risk alleles in the Indian and White T1D patients

A comparison of anthropometric and clinical characteristics with the number of *VDR* risk alleles in the Indian and White T1D patient is summarised in Table 3.15. There was no significant difference in the clinicopathological variables with an increasing number of *VDR* risk alleles in either the Indian or White T1D patients

Table 3.13: Comparison of the number of *VDR* risk alleles between T1D patients and controls in the Indian and White population

Number of risk alleles	Indian				White			
	T1D patients (n)	Control participants (n)	χ^2	p value (phi)	T1D patients (n)	Control participants (n)	χ^2	p value (phi)
1-2	2	14	9.190	0.027* (0.25)	24	17	0.315	0.872 (0.04)
3-4	30	32			27	24		
5-6	32	28			29	24		
7-8	9	7			8	6		

*p value is significant at $p < 0.05$

Table 3.14: Comparison of the number of vitamin D metabolising enzyme gene risk alleles between T1D patients and controls in the Indian and White population

Number of risk alleles	Indian				White			
	T1D patients (n)	Control participants (n)	χ^2	p value (phi)	T1D patients (n)	Control participants (n)	χ^2	p value (phi)
0	3	9	12.150	0.016* (0.28)	19	9	3.960	0.412 (0.15)
1	22	32			38	27		
2	26	27			32	35		
3	20	10			13	8		
4	4	0			2	2		

*p value is significant at $p < 0.05$

Table 3.15: Clinicopathological variables and the number of *VDR* gene risk alleles in the Indian and White T1D patients

Variables	Indian T1D patients[#]				White T1D patients				
	3-4 risk alleles (n = 30)	5-6 risk alleles (n = 32)	7-8 risk alleles (n = 9)	p value	1-2 risk alleles (n = 24)	3-4 risk alleles (n = 27)	5-6 risk alleles (n = 29)	7-8 risk alleles (n = 8)	p value
Age at diagnosis (years)	12.0 [10.0; 17.0]	13.0 [6.0; 17.0]	19.5 [15.0; 29.5]	0.718	15.0 [10.0; 20.0]	22.0 [10.0; 31.0]	19.5 [13.0; 30.0]	19.5 [15.0; 29.5]	0.157
BMI (kg/m^2)	24.4± 6.1	24.8 ± 7.7	23.1 ± 7.0	0.828	25.6 ±4.1	26.5 ± 3.9	24.6 ± 3.8	24.5 ± 5.2	0.281
Glucose (mmol/L)	9.9 [7.2; 12.1]	10.2 [6.8; 13.2]	9.0 [4.2; 13.0]	0.440	7.5 [6.2; 10.2]	7.0 [5.7; 11.0]	6.9 [5.8; 10.9]	9.0 [4.2; 13.0]	0.965
HbA1c (%)	8.6 [7.7; 9.7]	9.1 [8.1; 10.5]	8.2 [7.8; 9.1]	0.899	8.2 [7.0; 8.8]	7.9 [7.1; 8.7]	7.6 [7.0; 8.4]	8.2 [7.8; 9.1]	0.734
Vitamin D3 (nmol/L)	49.7 [27.8; 65.0]	56.0 [30.3; 72.0]	83.4 [53.2; 100.9]	0.530	82.1 [71.3; 92.8]	71.0 [59.9; 83.7]	75.4 [60.1; 95.1]	83.4 [53.2; 100.9]	0.651
Total vitamin D (nmol/L)	49.7 [27.8; 65.4]	56.0 [30.6; 76.3]	83.4 [53.2; 100.9]	0.449	82.1 [71.3; 92.8]	74.0 [64.2; 91.5]	76.7 [66.0; 95.7]	83.4 [53.0; 100.9]	0.627
ZnT8 AAb positivity (%, (n))	14.3 (4)	29.0 (9)	22.2 (2)	0.394	25.0 (6)	19.4 (7)	31.0 (9)	25.0 (2)	0.0763

*p value is significant at $p < 0.05$; HbA1c = glycated haemoglobin, BMI = body mass index, ZnT8 AAb = zinc transporter 8 autoantibodies, # data for the 1-2 risk alleles is not shown as there were only two participants and thus no statistical analyses could be performed with such a small number

3.7.2 Comparison of clinicopathological variables and the number of vitamin D metabolising gene risk alleles in the Indian and White T1D patients

A comparison of anthropometric and clinical characteristics with an increasing number of vitamin D metabolising gene risk alleles in the Indian and White T1D patient is summarised in Table 3.16. There were no significant differences in the clinicopathological variables and the number of vitamin D metabolising gene risk alleles in both the Indian and White T1D patients.

Table 3.16: Clinicopathological variables and the number of vitamin D metabolising gene risk alleles in the Indian and White T1D patients

Variables	Indian T1D patients				White T1D patients				
	0-1 risk# alleles (n = 25)	2 risk alleles (n = 26)	3-4 risk alleles (n = 24)	p value	0 risk alleles (n = 19)	1 risk alleles (n = 38)	2 risk alleles (n = 32)	3-4 risk# alleles (n = 15)	p value
Age at diagnosis (years)	14.5 [10.0; 23.0]	12.0 [6.0; 16.0]	14.0 [8.0; 19.0]	0.070	17.5 [9.0; 27.0]	20.0 [12.0; 29.0]	20.5 [13.0; 28.0]	19.0 [13.0; 25.0]	0.579
BMI (kg/m^2)	24.3 ± 8.0	24.9 ± 5.9	25.4 ± 6.5	0.861	26.7 ± 4.2	25.3 ± 3.7	25.1 ± 4.3	25.8 ± 3.8	0.583
Glucose (mmol/L)	10.2 [7.9; 13.2]	8.1 [6.7; 11.4]	8.3 [6.5; 10.8]	0.400	7..0 [4.8; 11.0]	7.5 [6.2; 10.9]	7.3 [4.8; 10.0]	8.5 [6.9; 13.1]	0.564
HbA1c (%)	9.1 [7.7; 9.9]	8.7 [7.9, 10.3]	9.3 [7.9; 9.8]	0.871	8.4 [7.1; 9.2]	7.7 [7.0; 8.2]	7.5 [7.0; 8.8]	8.2 [7.6; 8.8]	0.061
Vitamin D3 (nmol/L)	55.9 [30.6; 87.7]	36.6 [24.9; 63.4]	54.5 [34.4; 65.0]	0.516	78.7 [61.9; 98.8]	74.9 [60.1; 88.4]	72.2 [60.7; 92.6]	74.4 [67.0; 107.2]	0.970
Total vitamin D (nmol/L)	56.1 [30.6; 92.5	37.4 [28.3; 63.4]	56.5 [36.7; 72.0]	0.518	80.7 [69.6; 98.6]	77.9 [64.5; 94.5]	72.2 [60.7; 92.6]	88.8 [68.0; 107.2]	0.990
ZnT8 AAb positivity (%, (n))	20.0 (5)	29.2 (7)	16.7 (4)	0.269	27.8 (5)	23.7 (9)	25.0 (8)	20.0 (2)	0.960

*p value is significant at $p < 0.05$, HbA1c = glycated haemoglobin, BMI = body mass index, ZnT8 AAb = zinc transporter 8 autoantibodies, # risk allele data were combined due to small number of participants

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

A multiple linear regression model was run to investigate which variables contribute to vitamin D levels in the total population (Table 3.17). *VDR* and metabolising enzyme gene polymorphisms, patient/control, ethnicity, age, BMI, smoking, time outdoors, sun exposure and season of collection were included in this model. Indian race ($p < 0.001$) and *CYP2R1* AA/GA genotype ($p = 0.028$) were found to significantly contribute to vitamin D3 levels in the total population ($p < 0.001$ and 0.026, respectively). Indian race was associated with vitamin D3 levels 25.05 nmol/L lower than vitamin D levels in the White population. In addition, the *CYP2R1* GG genotype was associated with vitamin D3 levels approximately 8.29 nmol/L lower than levels in individuals with the GA or AA genotypes. This model accounts for 36.6% of the contributors to vitamin D levels.

Table 3.17: Variables associated with levels of vitamin D3 in the total population

Dependant variables	**Independent variable**	**b value**	**p value**	**R and (p) value for whole model**
Vitamin D3	Indian race	-25.05	<0.001*	0.366 (0.000)
	CYP2R1 AA/GA	8.29	0.026*	

*p value is significant at $p < 0.05$

When determining what variables contributed to total vitamin D levels in the total population (Table 3.18), only Indian race remained significantly associated ($p < 0.001$); with individuals of Indian ethnicity having vitamin D levels that were on average 24.13 nmol/L lower than those of White ethnicity. This model accounts for 34% of contributors to vitamin D levels.

Table 3.18: Variables associated with levels of total vitamin D in the total population

Dependant variables	Independent variable	b value	p value	R and (p) value for whole model
Total vitamin D	Indian race	-24.13	<0.001*	0.343 (0.000)

*p value is significant at $p < 0.05$

To determine population specific contributors to vitamin D levels, the regression model was repeated in each individual population group using the variables described above, with the exception of race. None of the variables contributed to serum vitamin D levels in the Indian population. However, in the White population (Table 3.19) the *CYP2R1* genotype was found to significantly contribute to both vitamin D3 and total vitamin D levels (p = 0.010 and 0.003, respectively). Within the White population, individuals with the *CYP2R1* GG genotype had vitamin D3 levels 13.19 nmol/L and total vitamin D levels 15.04 nmol/L lower than individuals with the AA/AG genotypes. These models account for approximately 20% of variables affecting both total vitamin and vitamin D3 levels.

Table 3.19: Variables associated with levels of vitamin D in the White population

Dependant variables	Independent variable	b value	p value	R and (p) value for whole model
Vitamin D3	*CYP2R1* AA/AG	13.19	0.010*	0.194 (0.000)
Total vitamin D	*CYP2R1* AA/AG	15.04	0.003*	0.223 (0.000)

*p value is significant at $p < 0.05$

3.9 Comparison of relative *VDR* mRNA expression levels between White T1D patients and control participants

A pilot study to compare *VDR* mRNA expression levels between White T1D patients (n = 7) and control participants (n = 9) was carried out (Table 3.20). Relative *VDR* mRNA expression was expressed as a ratio between the Ct of *VDR* and the Ct of the

β-actin reference gene. There was a significant difference in *VDR* mRNA expression levels between T1D patients and controls (0.88 ± 0.07 vs. 0.76 ± 0.074, p = 0.007). Representative amplification curves, melt curves and agarose gel electrophoresis of the real time assay are shown in Appendix 5.

Table 3.20: Comparison of *VDR* mRNA expression between White T1D patients and control participants

Gene	**White T1D patients**		**White control participants**		**p value**
	n	**mRNA expression#**	**n**	**mRNA expression#**	
VDR	7	0.88 ± 0.07	9	0.76 ± 0.07	0.007

*p value is significant at $p < 0.05$; # medium quantification of mRNA expression

4 Discussion

In this study we investigated the association of the *VDR* and vitamin D metabolising enzyme gene polymorphisms with T1D. The *VDR* gene polymorphisms *Bsm*I, *Apa*I and *CYP2R1* showed a significant difference in allele frequency between Indian T1D patients and controls. The A alleles of the *Bsm*I, *Apa*I and *CYP2R1* polymorphisms were found at higher frequencies in Indian T1D patients than control participants. In the White population, no significant difference was observed for any of the polymorphisms investigated between patients and controls, with the exception of the *CYP27B1* polymorphism, where the GG genotype/G allele frequencies were significantly higher in the patient group. In the White population, the majority (60.2%) of the participants had vitamin D levels greater than 72.5 nmol/L, whereas only 28.4% of Indian participants had sufficient levels. The main determinants of vitamin D levels in the total cohort was found to be ethnicity and *CYP2R1* GG/AG genotype.

4.1 Anthropometric and clinical characteristics of the White and Indian populations

In the White population, the control participants were significantly younger than T1D participants ($p < 0.001$). This can be explained by the fact that the majority of the White controls were students recruited from Wits Medical School and thus had a younger age. In contrast, in the Indian population, the T1D participants were younger than control participants ($p < 0.001$). This may be due to the fact that the majority of control participants were recruited from SANBS blood drives which may draw from an older population (personal observation of the recruiter).

The White T1D participants had a higher BMI ($p = 0.046$) and larger waist circumference ($p = 0.043$) than control participants. This is not unexpected as the White control group is significantly younger than the T1D participants and older age is known to be associated with increased BMI and waist circumference (Peter *et al*., 2015).

The White and Indian control group had lower glucose concentrations than T1D participants ($p < 0.001$). This finding is expected because T1D is a disease that results

in β cell destruction and hence low insulin levels (Denis Daneman, 2006). Insulin is necessary to regulate glucose levels in the blood, thus in the absence of insulin, hyperglycaemia is found (Joshi *et al*., 2007).

The higher percentage of males in the Indian control population may be due to the fact that they were recruited from SANBS blood drives. According to the SANBS 2017 integrated report, there is strong evidence that iron deficiency is more prevalent in female donors (SANBS, 2017), thus less women would be eligible to be blood donors. In addition, the World Health Organisation reported that globally, only 30% of blood donors are female (WHO, 2017).

In contrast, there was a higher percentage of females in the White control population. These discrepancies are likely due to the composition/nature of the participants approached to participate in this study. In addition, data from 2017 showed that there was a greater percentage of female students enrolled at WITS University compared to male students (54.9 vs. 45.1%) which could explain the greater number of female participants in the White control group (Wits University Report, 2018). Both the Indian and WhiteT1D patient groups showed a similar percentage of females as to males. Studies conducted by Kalk *et al*. (1993) and Omar and Asmal (1984) in South African populations showed a predominance of females. It is observed that a predominance of females in T1D is mostly seen in countries with a low incidence while the opposite is true in country with a high incidence such as Europe (Karvonen *et al*., 1997).

The majority of the Indian population (41.9%) were vitamin D deficient, while 29.7% had insufficient levels and 28.4% of participants had sufficient levels of vitamin D. This is in agreement with a study conducted by George *et al*. (2014) which showed that a South African Indian population had a higher percentage of vitamin D deficiency (28.6%) as compared to White South Africans.

The majority of the White population (60.2%) were vitamin D sufficient with 28.7% having insufficient levels and only 11.0% of participants being vitamin D deficient. This is similar to another study undertaken in Gauteng, which showed that the majority of the White population (70%) had sufficient vitamin D levels (O' Neill *et al*., 2013). The

similarities between the two studies is expected as both studies looked at a common population group, i.e. White participants recruited from Gauteng province.

4.2 Characteristics of the Indian and White T1D participants

4.2.1 Indian T1D patients have a younger age at diagnosis than White T1D patients

The White T1D patients had a significantly older median age at diagnosis than the Indian T1D patients (20.0 [11.0; 19.0] vs. 13.0 [8.0; 17.0] years; $p < 0.001$). When looking at the distribution for the age at diagnosis in the White T1D participants, two peaks were observed (11-15 years and 26-30 years), whereas a single peak at 11-15 years was observed in the Indian T1D population.

The peak age at diagnosis seen in our Indian cohort is similar to that described by Omar and Asmal (1984) who showed a peak age at diagnosis of 17 years in the Durban Indian population. Similarly, in South Indian populations, the peak age at diagnosis was 12-15 years (Mylariah and Kumar, 2015).

The first age at diagnosis peak seen in the White T1D patients corresponds to studies in South African White and European populations which found the age of diagnosis to be 12-13 years and 10-14 years, respectively (Patterson *et al*., 2009; Kalk *et al.,* 1993). A recent study conducted in the United Kingdom on the frequency of T1D in the first six decades of life observed that about 42% of their T1D participants were diagnosed between 31 and 60 years of age (Thomas *et al*., 2017). In our White cohort 18.3% were diagnosed between 31 and 60 years of age. This is in contrast to the Indian population where only 9.1 % of individuals were diagnosed after the age of 30 years. The UK study found that there were no differences in clinical characteristics between T1D cases diagnosed ≤30 years of age and those diagnosed at >30 years of age. However, when comparing T1D individuals diagnosed between 31-60 years of age with T2D patients, significant differences in clinical characteristics (BMI, use of insulin within a year of diagnosis and presence of ketoacidosis) were identified (Thomas *et al*., 2017). These differences in clinical characteristics suggest that the T1D participants with the older age at diagnosis are not misdiagnosed T2D patients.

Studies conducted in Italy and Lithuania showed an increasing incidence of T1D in all age groups, including those diagnosed at 40 years or older (Bruno *et al*., 2009; Pundziute-Lycka *et al*., 2003). These findings challenge the notion of a shift to an earlier age of onset and also reveal the increasing incidence of both childhood and adult onset T1D (Borchers *et al*., 2009). Thus, it is probable that the older age at diagnosis seen in our White T1D population is due to an increased incidence of T1D later in life as well as fewer late onset T1D participants being misclassified as T2D patients. It is possible, however that some of these late onset T1D participants may have latent autoimmune diabetes in adults (LADA), which is similar to late onset T1D, but has a slower disease progression (Stenström *et al*., 2005).

4.2.2 A low prevalence of ZnT8 AAb positivity in South African Indian and White T1D patients

ZnT8 AAbs are found in the majority (60-80%) of newly diagnosed White T1D patients (Wenzlau *et al*., 2007; Vaziri-Sani *et al*., 2010) whereas in the Indian population ZnT8 AAb positivity was shown to be 31.8% (irrespective of duration of disease). This AAb frequency increased to 45% in newly diagnosed T1D patients (Shivaprasad *et al*., 2014). In our cohort, 21.3% of the Indian and 23.6% of the White T1D patients were ZnT8 AAb positive. The ZnT8 AAb positivity in our Indian cohort was similar to that described by Shivaprasad and colleagues in an Indian study (21.3 vs. 31.8%; p= 0.122). In contrast, we found a much lower ZnT8 AAb positivity in the White South African T1D participants when compared to published literature (21.5 vs. 60%; p<0.001). The low percentage of AAb positivity in the White patients may be due to their long disease duration (21.8 ± 12.6 years). Wenzlau *et al.* (2010) showed that ZnT8 AAb positivity declined from 64% at disease onset to only 6.7% after a disease duration of 25 years. In addition, our White cohort did not contain any newly diagnosed participants which is significant as ZnT8 AAb positivity is known to decline after manifestation of clinical symptoms of the disease (Vaziri-Sani *et al*., 2010).

4.2.3 Indian T1D patients have poorer glucose control than White T1D patients

HbA1c is a measure used to determine long term glucose control in diabetic patients. HbA1c is formed by the exposure of haemoglobin A beta chain to plasma glucose and its synthesis is equivalent to the relative plasma glucose concentration (Stettler *et al*., 2000). Indian T1D patients had a higher HbA1c than the White population (8.8 [7.7; 10.0] vs. 7.9 [7.1; 8.7] %; $p<0.001$). Indian patients were recruited primarily from public hospitals whilst the White participants were recruited through private clinics. It is therefore possible that the discrepancy in the HbA1c results is due to less intensive education and less frequent contact between patient and doctor in public compared to private institutions. Factors such as economic status (Viana *et al*., 2016), interaction with healthcare practitioners and diabetes care access (Schwartz *et al*., 2010) have been associated with poor glycaemic control and adherence to diabetic treatment, especially in adolescents. The Indian T1D patients were also significantly younger than the White T1D patients which may further explain the poorer glycaemic control observed in the Indian patients. A study conducted on adolescents showed that poor glycaemic control is prevalent in this age group due to psychosocial and life stress (Goldston *et al*., 1995).

4.2.4 Indian T1D patients have lower vitamin D levels than White T1D patients

The lower levels of vitamin D seen in the Indian patients compared to the White patients is likely to be due to skin pigmentation, clothing choices and a lack of vitamin D supplementation (discussed in more detail in section 4.9).

4.3 Genotypic and allelic frequency of the *VDR* and vitamin D metabolising enzyme gene polymorphisms in the study population

We investigated the genotypic and allelic frequency of *VDR* and vitamin D metabolising enzyme gene polymorphisms associated with the development of T1D in the South African White and Indian populations. All the polymorphisms were in HWE with the exception of the *CYP27B1* polymorphism. Our cohort is thus not considered representative of the population as a whole for the *CYP27B1* polymorphism. A

possible reason for this is the relatively small sample size of the study. However, for the purpose of this dissertation the data for the *CYP27B1* polymorphism was included in all analyses.

4.3.1 *VDR* polymorphism allelic frequencies in South African Indian and White participants are not significantly different from population matched frequencies in the 1000 Genomes project.

The allele frequencies of the four *VDR* polymorphisms investigated in the Indian population were statistically similar to the South Asian populations in the 1000 Genome project. Similarly, in the White population, the VDR allele frequencies were not significantly different to those of the European populations in the 1000 genomes project. These findings are not unexpected as the majority of the Indian population in South Africa originate from South Asia (Anahita Mukherji, 2011), and the White population originates from Western Europe (John Hunt, 2005).

In addition, the frequencies of the *Apa*I and *Taq*I alleles in our Indian and White control participants was not significantly different to those reported by Ojwang *et al.* (2001) who conducted a study on healthy Indian and White blood donors in the Durban metropolitan area of South Africa.

4.3.2 The frequency of the *CYP27B1* polymorphism in the White South African population is significantly different to published literature

The frequency of the *CYP27B1* G allele did not differ between our Indian population and the 1000 Genome project South Asian populations. However, the frequency of the *CYP27B1* G allele in the White population was significantly lower than that seen in the 1000 Genome project European population. However, the *CYP27B1* polymorphism was not in HWE, which may explain why the results obtained in our study differ from that seen in the 1000 Genomes project. No significant differences in the *CYP2R1* allelic frequencies were found between the South African Indian and White populations and the South Asian and European populations, respectively in the 1000 Genomes project.

4.4 The association of *VDR* polymorphisms with T1D in the South African Indian and White population

4.4.1 The *Bsm*I and *Apa*I *VDR* polymorphisms are associated with T1D in the South African Indian population

Within the Indian cohort, the *Bsm*I and *Apa*I AA genotypes were found at higher frequencies in theT1D as compared to control participants. In addition, the *Bsm*I and *Apa*I A alleles were found at higher frequencies in the T1D group compared to the control population. Thus, the A allele for these two polymorphisms is associated with an increased risk of T1D in the South African Indian population. No significant differences in either genotypic or allelic frequencies were found between Indian T1D and control participants for the *Fok*I and *Taq*I polymorphisms. Thus, these polymorphisms are not associated with T1D in this South African Indian cohort.

The association of the *Bsm*I A allele with T1D in our Indian population is supported by studies carried out in Egyptian, Indian Asian and Greek populations that similarly showed that the *Bsm*I A allele is the risk allele for the development of T1D (Abd-Allah *et al*., 2014); McDermott *et al*., 1997); Panierakis *et al*., 2009). However, studies conducted on Taiwanese and Japanese populations found the *Bsm*I A allele at a lower frequency in T1D patients when compared to control participants (Chang *et al*., 2000; Motohashi *et al.,* 2003). A study conducted by Pani *et al*. (2000) on a German population showed the A allele to be protective. Conversely, studies conducted on Chilean, Portuguese, German, Japanese and Finnish populations showed that there was no difference in allelic frequency distribution between T1D patients and controls (Angel *et al*., 2004; Lamos *et al*., 2008; Nejentsev *et al*., 2004; Yokota *et al.,* 2002; Turpeinen *et al*., 2003).

Studies conducted on Egyptian, North Indian, Dalmatian and Greek populations which showed that the *Fok*I T allele frequency was significantly increased in patients thus, individuals with the T allele appeared to carry a higher risk of developing T1D (Abd-Allah *et al*., 2014; Israni *et al*., 2009; Zemunik *et al*., 2005; Panierakis *et al*., 2009). In contrast, a study by Ban *et al*. (2001) showed that the *Fok*I C allele was associated with T1D susceptibility in the Japanese population. Our observation in both the White

and Indian populations are similar to studies conducted in Portuguese, Iranian and Finnish populations (Lamos *et al*., 2008; Mohammadnejad *et al*., 2011; Turpeinen *et al*., 2003), where there was no association observed between the *Fok*I T allele polymorphism and T1D.

Studies conducted on Taiwanese and Greek populations showed that the *Apa*I A allele was more frequent in T1D patients as compared to control participants and is associated with an increased risk of T1D (Chang *et al*., 2000; Panierakis *et al*., 2009). These studies support our findings in the Indian population. In contrast, a study conducted on a German population showed that the *Apa*I A allele was protective (Pani *et al*., 2000). In addition, no association was found between the *Apa*I polymorphism and T1D in Chilean, Portuguese, German, Japanese and Finnish study populations (Angel *et al*., 2004; Lamos *et al*., 2008; Nejentsev *et al*., 2004; Turpeinen *et al*., 2003).

Studies conducted on Indian, Iranian and Romanian populations showed that the *Taq*I C allele increased the risk of T1D (McDermott *et al*., 1997; Mohammadnejad *et al*., 2011; Guja *et al*., 2002). However, a study conducted by Fassbender *et al*. (2002) on a German population showed a higher frequency of the *Taq*I T allele in T1D patients. The lack of an association of the *Taq*I polymorphism with T1D in our study cohort is supported by studies in the Chilean, Portuguese, and Finnish populations where they found no association between this polymorphism and T1D (Angel *et al*., 2004; Lamos *et al*., 2008 and Turpeinen *et al*., 2003).

The *Bsm*I and *Apa*I A alleles may alter the transcription rate of the *VDR* gene. A decreased transcription rate results in less VDR available for binding to the available vitamin D. This would in turn result in an increase in the transcription of the pro-inflammatory cytokine genes (IL-12, IL-2, INFγ) which subsequently lead to the destruction of the beta cells and development of T1D.

A possible reason as to why our results differ from other studies may be because the VDR polymorphisms studied are not the polymorphisms which are responsible for the changes in protein transcription and activity, but are in linkage disequilibrium with the polymorphisms responsible for the effect. Different population groups may have different alleles in linkage equilibrium with the disease causing variant.

4.4.2 The *VDR* polymorphisms are not associated with T1D in the South African White population

In the White population, there was no significant difference observed in the genotypic and allelic frequencies of the *VDR* gene polymorphism *Bsm*I, *Fok*I, *Apa*I and *Taq*I between T1D patients and controls.

4.5 The association of vitamin D metabolising gene polymorphisms with T1D in the South African Indian and White population

4.5.1 The *CYP2R1* polymorphism is associated with T1D in the South African Indian population

A statistically significant difference in the genotypic and allelic frequencies for the *CYP2R1* polymorphism between Indian patient and control groups was seen with the A allele associated with T1D. This finding is in contrast with a study conducted by Ramos-Lopez *et al*. (2007) on German individuals which showed that the G allele was associated with susceptibility to T1D. The G allele of *CYP2R1* results in reduced enzyme activity of 25-hydroxylase. As *CYP2R1* is responsible for the hydroxylation of vitamin D into 25(OH)D_3, a decrease in enzymatic activity is associated with a reduced concentration of 25(OH)D_3 available to be hydroxylated by *CYP27B1* into the active 1,25$(OH)_2D_3$ (Cheng *et al*., 2014). Thus the *CYP2R1* G allele results in a decreased amount of the biologically active form of vitamin D. The reason our results differ from other studies may be because the *CYP2R1* polymorphism is not the polymorphism which is responsible for the changes in enzyme activity, but is in linkage disequilibrium with the polymorphism which is responsible for the effect. Different population groups may have different alleles in linkage equilibrium with the disease causing variant.

We found no association between the genotypic and allelic frequencies for the *CYP27B1* polymorphism studied between the T1D and control participants in the Indian population.

4.5.2 The *CYP27B1* polymorphism is associated with T1D in the South African White population

Within the White cohort, the *CYP27B1* polymorphism showed a significant difference in the genotypic and allelic frequencies between T1D patients and control participants. For this polymorphism, the GG genotype/G allele was associated with T1D. However, as this polymorphism was not in HWE, the results we obtained are not reliable. However, the GG genotype/ G allele results in decreased *CYP27B1* mRNA expression G allele (Ramos-Lopez *et al*., 2007; Amit *et al*., 2014). Reduced mRNA is thought to results in decreased protein expression and thus less enzyme available to metabolise vitamin D to its active form. Thus whilst we cannot be certain of these results, it does match with the literature.

A study conducted on a German population showed the *CYP27B1* G allele to be at a higher frequency in T1D patients and the GG genotype was associated with decreased expression levels of *CYP27B1* mRNA (Ramos-Lopez *et al*., 2004, Ramos-Lopez *et al*., 2007). Hussein *et al*. (2012) showed that there is a 3.7 fold increased risk of developing T1D patients with the *CYP27B1* GG genotype.

No statistically significant differences in the genotypic and allelic frequencies were found between patients and controls for the *CYP2R1* polymorphism in the White population.

The *CYP2R1* polymorphism findings in our White population are similar to a Polish study and a pilot study in Germany where they failed to show any association between the vitamin D metabolising enzyme gene polymorphisms and T1D as there was no difference in the distribution of genotypic and allelic frequencies in patients and controls (Fichna *et al*., 2010; Rose *et al*., 2013).

4.6 *Apa*I genotypes contribute to differences in clinicopathological variables in Indian T1D participants

Indian T1D patients with the *Apa*I CC/CA genotype had a significantly older age at diagnosis and higher glucose levels when compared to patients with the AA genotype

(p = 0.018 and p = 0.035). This is in contrast to a study conducted by McDermott *et al.* (1997) in an Indian Asian population. In this study, they did not observe a difference between age at diagnosis and the genotypic distribution of *Apa*I (p = 0.90), *Bsm*I (p = 0.87) and *Taq*I (p = 0.84) in T1D patients diagnosed at ≤15 or ≥ 15 years of age. In our study, when patients were split into two groups based on their age at diagnosis i.e. those diagnosed at ≤15 or ≥ 15 years of age, no significant difference was observed between *Apa*I genotypes and age at diagnosis (p = 0.060). This matches the findings in the McDermott study. It is possible that if the McDermott study had analysed their data as continuous rather than categorical data as we did, they might have observed similar results.

A study conducted on a Japanese population showed that the *Fok*I C allele was associated with a younger age at diagnosis as compared to patients with the T allele. However, no association was found between *Apa*I and *Taq*I genotypes and age at diagnosis (Yokota *et al*., 2002). The discrepant results in the above mentioned studies may be due to the different sample sizes in each study and selection of a specific age at diagnosis cut-off (0.4-18 years; Yokota *et al*., 2002). In addition, the conflicting results could be due to the different ethnic groups that the studies were conducted in.

4.7 *VDR* and vitamin D metabolising enzyme gene polymorphisms are not associated with lower levels of vitamin D in South African Indian and White T1D patients

No significant difference was observed between the *VDR* gene polymorphisms and vitamin D levels in either the Indian or White population. Since *Bsm*I, ApaI and *Taq*I are non-coding variants, they are not expected to affect the structure of the *VDR* protein. However, they may result in different transcription rates of the VDR and thus slower uptake of vitamin D into the cell despite normal levels of vitamin D. Alternatively, it is possible that serum levels of vitamin D showed no association with *VDR* genotypes within our cohort as individuals may have been on vitamin D supplementation giving falsely elevated vitamin D concentrations.

No significant difference was observed between the vitamin D metabolising enzyme gene polymorphisms and vitamin D levels in either the Indian or White population.

These polymorphisms have been observed to have an effect on enzymatic activity of 25-hydroxylase and 1 α-hydroxylase resulting in lower concentrations of vitamin D and hence T1D. However, it is possible that it is in fact the presence of another polymorphism, in linkage disequilibrium with the polymorphism that is causing this effect. This would explain why we do not see an association of these polymorphisms with T1D in our cohort. Alternatively, the lack of association may be due to the sample size being too small.

4.8 An increasing number of *VDR* and vitamin D metabolising enzyme gene polymorphism risk alleles are associated with T1D in the Indian but not White population

Allele risk scores combine multiple genetic variants that are associated with a specific risk factor of a disease (Burgess and Thompson, 2013). The Indian population showed that an increasing number of *VDR* gene risk alleles is associated with T1D, where control participants had fewer risk alleles than T1D patients ($p = 0.027$). Similarly, there was an association between the number of vitamin D metabolising enzyme gene risk alleles and T1D in the Indian population ($p = 0.016$). However, there was no association between the number of *VDR or* vitamin D metabolising enzyme gene risk alleles and T1D in the White population.

In both the Indian and White T1D patients, there was no significant difference between clinicopathological variables and an increasing number of *VDR* or vitamin D metabolising enzyme gene risk alleles.

Interestingly, vitamin D levels tended to increase with an increasing number of *VDR* risk alleles. This is contrary to what one would expect if low levels of vitamin D are associated with the development of T1D and further investigations are warranted to explain this finding.

4.9 Indian ethnicity and the *CYP2R1* GG genotype contribute to lower vitamin D levels

In the total study population, Indian ethnicity and *CYP2R1* genotype were observed to be independent contributors to vitamin D levels ($p = 0.000$ and $p = 0.026$, respectively). We observed that individuals of Indian ethnicity had 24 - 25 nmol/L less vitamin D levels than the White population. The reason the Indian population has lower vitamin D levels might be due to skin pigmentation and skin coverage due to their traditional clothing style reducing vitamin D synthesis in the skin. Skin pigmentation and dress code are some of the factors that are thought to affect vitamin D synthesis (Harinarayan and Joshi, 2009). Another reason for the difference in vitamin D levels might be that the some of the participants may be taking multivitamins which contain vitamin D3 or taking vitamin D3 supplements which may account for the differences in vitamin D levels observed. Individuals with the *CYP2R1* GG genotype had 8.29 nmol/L lower vitamin D levels than individuals with the AA/AG genotype. It is likely that the *CYP2R1* polymorphism remains significantly associated with both total serum vitamin D levels (combined vitamin D3 and D2) and vitamin D3 as 25 hydroxylase hydroxylates both vitamin D2 and D3 to $25(OH)D_3$ and thus would have an effect of vitamin D levels irrespective of supplementation.

In the White population only the *CYP2R1* AA/GA genotype was found to be an independent contributor to vitamin D levels ($p = 0.010$). Individuals with the *CYP2R1* GG genotype had vitamin D levels 13-15 nmol/L lower than individuals with the AA/GA genotypes.

These findings are similar to a study conducted on Germans where T1D patients with the *CYP2R1* GG or GA genotype had lower vitamin D levels than those with the AA genotype (Ramos-Lopez *et al*., 2007). *CYP2R1* has an effect on vitamin D levels because the *CYP2R1* G allele results in a change in 25-hydroxylase enzyme activity which then results in low levels or lack of $25(OH)D_3$ leading to T1D.

4.10 *VDR* mRNA expression is higher in T1D patients when compared to control participants

This experiment was conducted on a very small sample therefore the results might not be representative of the whole population and no definite conclusion can be drawn. However, despite the small sample a significant difference was observed in the *VDR* mRNA expression levels between T1D patients and controls ($p = 0.007$). Since this was a pilot study, the study will need to be conducted on a larger sample size to see if the effect of the observed difference will still be present. There was greater mRNA expression levels in T1D patients. This was unexpected as increased VDR is expected to result in increased transfer of vitamin D into the cell and enhanced initiation of the VDR-RXR-vitamin D signalling cascade which promotes transcription of the anti-inflammatory cytokines that inhibit beta cell destruction. However, it is possible that the mRNA levels do not match the protein levels and thus western blot analysis would be required to confirm this finding. Furthermore, increased mRNA stability has been associated with the *Fok*I and *Taq*I C allele (Ogunkolade *et al*., 2002). However, this association was not replicated in our study as we did not observe any differences in allelic frequencies associated with mRNA stability between the T1D patients and controls. Our findings are in agreement with studies conducted on *VDR* polymorphisms and mRNA stability which showed that the polymorphisms did not influence mRNA stability (Gross *et al*., 1998; Durrin *et al*., 1999; Verbeek *et al*., 1997). Therefore, the observed differences in the mRNA expression is not due to the presence of the alleles associated with mRNA stability.

4.11 Limitations of the study

One of the limitations of the study is that was carried out on a relatively small sample. This may have prevented the detection of small associations of the polymorphisms with both serum vitamin D levels as well as T1D. In addition, the small sample size might explain why the *CYP27B1* polymorphism was not in HWE.

Not all patients/controls were matched across season of collection, age and BMI which may have altered comparative vitamin D levels and influenced the outcome of the

study. In addition, it is unknown how many participants were taking vitamin D supplementation and did not declare this.

Whilst all of the participants were recruited based on a clinical diagnosis of T1D (i.e. started on insulin treatment within six months of diagnosis), it is possible that some patients were in fact LADA patients and not true T1D patients. To confirm this, we would need to screen patients for GAD65 AAbs.

This study did not look at whether the polymorphisms within our study were in linkage disequilibrium with each other or with any other polymorphisms within the *VDR*, *CYP2R1* and *CYP27B1* genes. In addition, this study did not address haplotypes.

Finally, time constraints prevented a more comprehensive study into *VDR* mRNA expression levels in these populations and prevented elimination of primer dimers for a more accurate assay.

Thus future studies would entail the recruitment of additional participants paying particular attention to matching patients and controls for both BMI, age and season of recruitment. In addition, the completion of the mRNA expression studies in both population groups should be continued with larger numbers and perhaps the use of a probe based assay rather than the current SYBR green assay. In addition, other polymorphisms on the *VDR* and/or those of the vitamin D binding protein genes and their association with T1D can be determined.

5 Conclusions

In conclusion, our study showed that the A alleles of the *VDR Bsm*I and *Apa*I polymorphisms and the *CYP2R1* vitamin D metabolising enzyme gene polymorphism are associated with the development of T1D in the South African Indian population. Our study also showed that an increasing number of risk alleles are associated with T1D in the Indian population.

We did not see an association with vitamin D levels and T1D in either the Indian or White cohorts. However, *CYP2R1* genotype and ethnicity were shown to be determinants of vitamin D levels. We did not see differences in vitamin D levels across the *VDR* and vitamin D metabolising enzyme gene genotypes in both the Indian and White cohort.

ZnT8 AAb positivity was lower (especially in our White T1D patients) than that reported elsewhere. This may be due to the lack of newly diagnosed patients in our cohort as it is known that ZnT8 AAb positivity declines with increasing duration of disease. In addition, it may be due to the fact that we saw a second, much later, peak for the age at diagnosis in this cohort. ZnT8 AAb titres are lower in patients with an older age at diagnosis (Vaziri-Sani *et al*., 2010) and it is possible that they were too low to detect in our patient group with the older age at diagnosis. It would be interesting to look at GAD65 and IA-2 AAb positivity in this cohort.

With an increasing number of studies implicating low levels of vitamin D with T1D and other diseases, intervention may be appropriate. Health care workers should consider recommending vitamin D testing and supplementation for individuals who may be at risk of vitamin D insufficiency and deficiency (i.e. infants, T1D patients, the elderly, dark skinned individuals and individuals who have limited sun exposure).

6 References

1. Abd-Allah SH, Pasha HF, Hagrass HA, Alghobashy AA. Vitamin D status and vitamin D receptor gene polymorphisms and susceptibility to type 1 diabetes in Egyptian children. Gene. 2014;536(2):430-4.
2. Almawi WY, Tamim H, Azar ST. T Helper Type 1 and 2 Cytokines Mediate the Onset and Progression of Type I (Insulin-Dependent) Diabetes. The Journal of Clinical Endocrinology & Metabolism. 1999;84(5):1497-502.
3. Amit V, Vandana K, Neeraj K, Preeti K. Vitamin D and diabetes: A new horizon. Acta Medica International. 2014;1(2):134.
4. Angel B, Santos JL, Carrasco E, Albala C, Pérez-Bravo F. Vitamin D receptor polymorphism and susceptibility to type 1 diabetes in chilean subjects: A case-parent study. European Journal of Epidemiology. 2004;19(12):1085-7.
5. Arneil G. Nutritional rickets in children in Glasgow. The Proceedings of the Nutrition Society. 1975;34(2):101-9.
6. Arya V, Bhambri R, Godbole MM, Mithal A. Vitamin D status and its relationship with bone mineral density in healthy Asian Indians. Osteoporosis International. 2004;15(1):56-61.
7. Atkinson MA. The pathogenesis and natural history of type 1 diabetes. Cold Spring Harbor perspectives in medicine. 2012;2(11):a007641.
8. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet. 2001;358(9277):221-9.
9. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. The Lancet. 2014;383(9911):69-82.
10. Atkinson MA, MacLaren NK. Mechanisms of disease: the pathogenesis of insulin-dependent diabetes mellitus. N Engl J Med. 1994;331.
11. Azar ST, Tamim H, Beyhum HN, Habbal MZ, Almawi WY. Type I (insulin-dependent) diabetes is a Th1-and Th2-mediated autoimmune disease. Clin Diagn Lab Immunol. 1999;6(3):306-10.
12. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. Current Opinion in Pharmacology. 2010;10(4):482-96.
13. Bækkeskov S, Warnock G, Christie M, Rajotte RV, Larsen PM, Fey S. Revelation of specificity of 64K autoantibodies in IDDM serums by high-

resolution 2-D gel electrophoresis: unambiguous identification of 64K target antigen. Diabetes. 1989;38(9):1133-41.

14. Bailey R, Cooper JD, Zeitels L, Smyth DJ, Yang JH, Walker NM, et al. Association of the vitamin D metabolism gene *CYP27B1* with type 1 diabetes. Diabetes. 2007;56(10):2616-21.
15. Ban Y, Taniyama M, Yanagawa T, Yamada S, Maruyama T, Kasuga A, et al. Vitamin D receptor initiation codon polymorphism influences genetic susceptibility to type 1 diabetes mellitus in the Japanese population. BMC Medical Genetics. 2001;2(1):7.
16. Barker JM, Barriga KJ, Yu L, Miao D, Erlich HA, Norris JM, et al. Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). The Journal of Clinical Endocrinology & Metabolism. 2004;89(8):3896-902.
17. Belle TLV, Coppieters KT, Herrath MGV. Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies. Physiological Reviews. 2011;91(1):79-118.
18. Beydoun MA, Boueiz A, Shroff M, Beydoun H, Wang Y, Zonderman A. Associations among 25-hydroxyvitamin D, diet quality, and metabolic disturbance differ by adiposity in adults in the United States. The Journal of Clinical Endocrinology & Metabolism. 2010;95(8):3814-27.
19. Bikle DD. Vitamin D and immune function: understanding common pathways. Current osteoporosis reports. 2009;7(2):58.
20. Boldison J, Wong FS. Immune and Pancreatic beta Cell Interactions in Type 1 Diabetes. Trends in endocrinology and metabolism: TEM. 2016;27(12):856-67.
21. Borchers AT, Uibo R, Gershwin ME. The geoepidemiology of type 1 diabetes. Autoimmunity reviews. 2010;9(5):A355-A65.
22. Borges MC, Martini LA, Rogero MM. Current perspectives on vitamin D, immune system, and chronic diseases. Nutrition. 2011;27(4):399-404.
23. Brekke HK, Ludvigsson J. Vitamin D supplementation and diabetes-related autoimmunity in the ABIS study. Pediatric diabetes. 2007;8(1):11-4.
24. Bruno G, Runzo C, Cavallo-Perin P, Merletti F, Rivetti M, Pinach S, et al. Incidence of type 1 and type 2 diabetes in adults aged 30–49 years: the population-based registry in the province of Turin, Italy. Diabetes care. 2005;28(11):2613-9.

25. Busta A, Alfonso B, Poretsky L. Role of vitamin D in the pathogenesis and therapy of type 1 diabetes mellitus. Type 1 Diabetes-Complications, Pathogenesis, and Alternative Treatments: IntechOpen; 2011.
26. Chang T-J, Lei H-H, Yeh J-I. Vitamin D receptor gene polymorphisms influence susceptibility to type 1 diabetes mellitus in the Taiwanese population. Clinical endocrinology. 2000;52.
27. Chen TC, Holick MF. Vitamin D deficiency: a worldwide problem with health consequences. The American Journal of Clinical Nutrition. 2008;87(4):1080S-6S.
28. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human *CYP2R1* enzyme is a key vitamin D 25-hydroxylase. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(20):7711-5.
29. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of Cytochrome P450 2R1 a microsomal vitamin D 25-hydroxylase. Journal of Biological Chemistry. 2003;278(39):38084-93.
30. Christakos S. Mechanism of action of 1, 25-dihydroxyvitamin D 3 on intestinal calcium absorption. Reviews in Endocrine and Metabolic Disorders. 2012;13(1):39-44.
31. Cooper JD, Smyth DJ, Walker NM, Stevens H, Burren OS, Wallace C, et al. Inherited variation in vitamin D genes is associated with predisposition to autoimmune disease type 1 diabetes. Diabetes. 2011;60(5):1624-31.
32. D Schwartz D, D Cline V, A Hansen J, E Axelrad M, J Anderson B. Early risk factors for nonadherence in pediatric type 1 diabetes: a review of the recent literature. Current diabetes reviews. 2010;6(3):167-83.
33. DAHLQUIST G, MUSTONEN L. Childhood Onset Diabetes—Time Trends and Climatological Factors. International Journal of Epidemiology. 1994;23(6):1234-41.
34. Dahlquist GG, Patterson C, Soltesz G. Perinatal risk factors for childhood type 1 diabetes in Europe. The EURODIAB Substudy 2 Study Group. Diabetes care. 1999;22(10):1698-702.
35. Daneman D. Type 1 diabetes. The Lancet. 2006;367(9513):847-58.
36. Davies JL, Kawaguchi Y, Bennett ST. A genome-wide search for human type 1 diabetes susceptibility genes. Nature. 1994;371.

37. Deluca HF. VITAMIN D METABOLISM. Clinical endocrinology. 1977;7(s1):1s-17s.
38. Deurenberg P, Deurenberg-Yap M. Differences in body-composition assumptions across ethnic groups: practical consequences. Current Opinion in Clinical Nutrition & Metabolic Care. 2001;4(5):377-83.
39. Deurenberg P, Yap MD, Wang J, Lin F, Schmidt G. The impact of body build on the relationship between body mass index and percent body fat. International Journal of Obesity. 1999;23(5):537.
40. Dunnigan M, Paton J, Haase S, McNicol G, Gardner MD, Smith CM. Late rickets and osteomalacia in the Pakistani community in Glasgow. Scottish Medical Journal. 1962;7(4):159-67.
41. Durrin LK, Haile RW, Ingles SA, Coetzee GA. Vitamin D receptor 3′-untranslated region polymorphisms: lack of effect on mRNA stability. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 1999;1453(3):311-20.
42. Faraco JH, Morrison NA, Baker A, Shine J, Frossard PM. *Apa*I dimorphism at the human vitamin D receptor gene locus. Nucleic acids research. 1989;17(5):2150.
43. Fassbender W, Goertz B, Weismüller K, Steinhauer B, Stracke H, Auch D, et al. VDR gene polymorphisms are overrepresented in German patients with type 1 diabetes compared to healthy controls without effect on biochemical parameters of bone metabolism. Hormone and metabolic research. 2002;34(06):330-7.
44. Ferrari S, Rixxoli R, Manen D, Slosman D, Bonjour JP. Vitamin D receptor gene start codon polymorphisms (*Fok*I) and bone mineral density: interaction with age, dietary calcium, and 3'-end region polymorphism. J Bone Miner Res. 1998;13.
45. Fichna M, Żurawek M, Januszkiewicz-Lewandowska D, Fichna P, Nowak J. PTPN22, PDCD1 and *CYP27B1* polymorphisms and susceptibility to type 1 diabetes in Polish patients. International journal of immunogenetics. 2010;37(5):367-72.
46. Field LL, Tobias R, Magnus T. A locus on chromosome 15q26 (IDDM3) produces susceptibility to insulin dependent diabetes mellitus. Nat Genet. 1994;8.

47. Fleet JC, Harris SS, Wood RJ, Dawson-Hughes B. The *Bsm*I vitamin D receptor restriction fragment length polymorphism (BB) predicts low bone density in premenopausal black and white women. J Bone Miner Res. 1995;10.
48. Fronczak CM, Barón AE, Chase HP, Ross C, Brady HL, Hoffman M, et al. In Utero Dietary Exposures and Risk of Islet Autoimmunity in Children. Diabetes Care. 2003;26(12):3237-42.
49. Gallagher JC, Sai AJ. Vitamin D insufficiency, deficiency, and bone health. Oxford University Press; 2010.
50. Garland CF, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB, et al. The Role of Vitamin D in Cancer Prevention. American Journal of Public Health. 2006;96(2):252-61.
51. George JA, Norris SA, van Deventer HE, Pettifor JM, Crowther NJ. Effect of adiposity, season, diet and calcium or vitamin D supplementation on the vitamin D status of healthy urban African and Asian-Indian adults. British Journal of Nutrition. 2014;112(4):590-9.
52. Gross C, Eccleshall T, Malloy P, Villa M, Marcus R, Feldman D. The presence of a polymorphism at the translation initiation site of the vitamin D receptor is associated with low bone mineral density in postmenopausal Mexican-American women. J Bone Miner Res. 1996;11.
53. Gross C, Musiol IM, Eccleshall TR, Malloy PJ, Feldman D. Vitamin D receptor gene polymorphisms: analysis of ligand binding and hormone responsiveness in cultured skin fibroblasts. Biochemical and biophysical research communications. 1998;242(3):467-73.
54. Group NCDS, Joner G, Stene LC. Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. The American Journal of Clinical Nutrition. 2003;78(6):1128-34.
55. Guja C, Marshall S, Welsh K, Merriman M, Smith A, Todd J, et al. The study of CTLA-4 and vitamin D receptor polymorphisms in the Romanian type 1 diabetes population. Journal of cellular and molecular medicine. 2002;6(1):75-81.
56. Gujral JS, McNally PG, Botha JL, Burden AC. Childhood-onset diabetes in the white and South Asian population in Leicestershire, UK. Diabet Med. 1994;11.
57. Gyorffy B, Vasarhelyi B, Krikovszky D, Madacsy L, Tordai A, Tulassay T, et al. Gender-specific association of vitamin D receptor polymorphism combinations

with type 1 diabetes mellitus. European journal of endocrinology. 2002;147(6):803-8.

58. Hanis CL, Boerwinkle E, Chakraborty R. A genome-wide search for human non-insulin dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. Nat Genet. 1996;13.
59. Harinarayan C, Ramalakshmi T, Prasad U, Sudhakar D. Vitamin D status in Andhra Pradesh: a population based study. Indian Journal of Medical Research. 2008;127(3):211.
60. Harinarayan CV. Prevalence of vitamin D insufficiency in postmenopausal south Indian women. Osteoporosis International. 2005;16(4):397-402.
61. Harinarayan CV. Vitamin D and diabetes mellitus. Hormones. 2014;13(2):163-81.
62. Harinarayan CV, Ramalakshmi T, Prasad UV, Sudhakar D, Srinivasarao PV, Sarma KV, et al. High prevalence of low dietary calcium, high phytate consumption, and vitamin D deficiency in healthy south Indians. The American journal of clinical nutrition. 2007;85(4):1062-7.
63. Harris SS. Vitamin D in type 1 diabetes prevention. The Journal of nutrition. 2005;135(2):323-5.
64. Haussler MR, Whitfield GK, Haussler CA. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res. 1998;13.
65. Hawa M, Rowe R, Lan MS, Notkins AL, Pozzilli P, Christie MR, et al. Value of Antibodies to Islet Protein Tyrosine Phosphatase–Like Molecule in Predicting Type 1 Diabetes. Diabetes. 1997;46(8):1270-5.
66. Heras P, Mantzioros M, Mendrinos D, Heras V, Hatzopoulos A, Xourafas V, et al. Autoantibodies in Type 1 diabetes. Diabetes Research and Clinical Practice.90(2):e40-e2.
67. Hill NJ, Van Gunst K, Sarvetnick N. Th1 and Th2 pancreatic inflammation differentially affects homing of islet-reactive CD4 cells in nonobese diabetic mice. The Journal of Immunology. 2003;170(4):1649-58.
68. Holick MF. Vitamin D: A millenium perspective. Journal of cellular biochemistry. 2003;88(2):296-307.
69. Holick MF. Vitamin D deficiency. New England Journal of Medicine. 2007;357(3):266-81.

70. Holick MF. Vitamin D and sunlight: strategies for cancer prevention and other health benefits. Clinical Journal of the American Society of Nephrology. 2008;3(5):1548-54.
71. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of Clinical Endocrinology & Metabolism. 2011;96(7):1911-30.
72. Holmes A, Enoch B, Taylor J, Jones M. Occult rickets and osteomalacia amongst the Asian immigrant population. QJM: An International Journal of Medicine. 1973;42(1):125-.
73. Humble MB, Gustafsson S, Bejerot S. Low serum levels of 25-hydroxyvitamin D (25-OHD) among psychiatric out-patients in Sweden: relations with season, age, ethnic origin and psychiatric diagnosis. The Journal of steroid biochemistry and molecular biology. 2010;121(1-2):467-70.
74. Hummel M, Bonifacio E, Schmid S, Walter M, Knopff A, Ziegler A-G. Brief communication: early appearance of islet autoantibodies predicts childhood type 1 diabetes in offspring of diabetic parents. Annals of internal medicine. 2004;140(11):882-6.
75. Hunt J. Dutch South Africa: early settlers at the Cape, 1652-1708: Troubador Publishing Ltd; 2005.
76. Hussein AG, Mohamed RH, Alghobashy AA. Synergism of *CYP2R1* and *CYP27B1* polymorphisms and susceptibility to type 1 diabetes in Egyptian children. Cellular immunology. 2012;279(1):42-5.
77. Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet. 2001;358(9292):1500-3.
78. Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, et al. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes care. 2015;38(10):1964-74.
79. Isaia G, Giorgino R, Rini G, Bevilacqua M, Maugeri D, Adami S. Prevalence of hypovitaminosis D in elderly women in Italy: clinical consequences and risk factors. Osteoporosis International. 2003;14(7):577-82.
80. Islam MZ, Akhtaruzzaman M, Lamberg-Allardt C. Hypovitaminosis D is

common in both veiled and nonveiled Bangladeshi women. Asia Pacific journal of clinical nutrition. 2006;15(1):81.

81. Israni N, Goswami R, Kumar A, Rani R. Interaction of vitamin D receptor with HLA DRB1* 0301 in type 1 diabetes patients from North India. PloS one. 2009;4(12):e8023.
82. Jones G, Strugnell SA, DeLUCA HF. Current understanding of the molecular actions of vitamin D. Physiological reviews. 1998;78(4):1193-231.
83. Joshi SR, Parikh RM, Das A. Insulin-history, biochemistry, physiology and pharmacology. Journal-Association of Physicians of India. 2007;55(L):19.
84. Kahn HS, Morgan TM, Case LD, Dabelea D, Mayer-Davis EJ, Lawrence JM, et al. Association of Type 1 Diabetes With Month of Birth Among U.S. Youth. The SEARCH for Diabetes in Youth Study. 2009;32(11):2010-5.
85. Kamen DL, Tangpricha V. Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. Journal of Molecular Medicine. 2010;88(5):441-50.
86. Kanis J. Vitamin D metabolism and its clinical application. The Journal of bone and joint surgery British volume. 1982;64(5):542-60.
87. Karvonen M, Pitkäniemi M, Pitkäniemi J, Kohtamäki K, Tajima N, Tuomilehto J. Sex difference in the incidence of insulin-dependent diabetes mellitus: an analysis of the recent epidemiological data. Diabetes/metabolism reviews. 1997;13(4):275-91.
88. Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Åkerblom HK. Environmental Triggers and Determinants of Type 1 Diabetes. Diabetes. 2005;54(suppl 2):S125-S36.
89. Kongsbak M, Levring TB, Geisler C, Von Essen MR. The vitamin d receptor and T cell function. Frontiers in immunology. 2013;4:148.
90. Kukreja A, Maclaren NK. Autoimmunity and Diabetes. The Journal of Clinical Endocrinology & Metabolism. 1999;84(12):4371-8.
91. Kumar KMP. Incidence trends for childhood type 1 diabetes in India. Indian journal of endocrinology and metabolism. 2015;19(Suppl 1):S34.
92. Kumar P, Krishna P, Reddy SC, Gurappa M, Aravind S, Munichoodappa C. Incidence of type 1 diabetes mellitus and associated complications among children and young adults: results from Karnataka Diabetes Registry 1995-2008. Journal of the Indian Medical Association. 2008;106(11):708-11.

93. Lehmann B, Meurer M. Vitamin D metabolism. Dermatologic Therapy. 2010;23(1):2-12.
94. Lemos MC, Fagulha A, Coutinho E, Gomes L, Bastos M, Barros L, et al. Lack of association of vitamin D receptor gene polymorphisms with susceptibility to type 1 diabetes mellitus in the Portuguese population. Human immunology. 2008;69(2):134-8.
95. Liblau RS, Singer SM, McDevitt HO. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunology today. 1995;16(1):34-8.
96. Lopez ER, Regulla K, Pani MA, Krause M, Usadel K-H, Badenhoop K. *CYP27B1* polymorphisms variants are associated with type 1 diabetes mellitus in Germans. The Journal of steroid biochemistry and molecular biology. 2004;89:155-7.
97. Malabanan A, Veronikis I, Holick M. Redefining vitamin D insufficiency. The Lancet. 1998;351(9105):805-6.
98. Mathieu C, Gysemans C, Giulietti A, Bouillon R. Vitamin D and diabetes. Diabetologia. 2005;48(7):1247-57.
99. Mathieu C, Laureys J, Sobis H, Vandeputte M, Waer M, Bouillon R. 1,25-dihydroxyvitamin D3 prevents insulitis in NOD mice. Diabetes. 1992;41.
100. Matsuda A, Kuzuya T, Iwamoto Y. HLA DR antigens in adult-onset and juvenile-onset Japanese insulin-dependent diabetic patients. Diabetes Res Clin Pract. 1988;5.
101. McDermott MF, Ramachandran A, Ogunkolade BW, Aganna E, Curtis D, Boucher BJ, et al. Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians. Diabetologia. 1997;40(8):971-5.
102. McKenna MJ. Differences in vitamin D status between countries in young adults and the elderly. The American journal of medicine. 1992;93(1):69-77.
103. Mehra NK, Kumar N, Kaur G, Kanga U, Tandon N. Biomarkers of susceptibility to type 1 diabetes with special reference to the Indian population. Indian Journal of Medical Research. 2007;125(3):321.
104. Mein CA, Esposito L, Dunn MG. A search for type 1 diabetes susceptibility genes in families from the United Kingdom. Nat Genet. 1998;19.
105. Menon P, Viramani A, Shah P, Raju R, Sethi A, Sethia S, et al. Childhood onset diabetes mellitus in India: an overview. International Journal of Diabetes in

Developing Countries. 1990;10:11-6.

106. Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2004;50(1):72-7.

107. Mohammadnejad Z, Ghanbari M, Ganjali R, Afshari JT, Heydarpour M, Taghavi SM, et al. Association between vitamin D receptor gene polymorphisms and type 1 diabetes mellitus in Iranian population. Molecular Biology Reports. 2012;39(2):831-7.

108. Mokry LE, Ross S, Ahmad OS, Forgetta V, Smith GD, Leong A, et al. Vitamin D and risk of multiple sclerosis: a Mendelian randomization study. PLoS medicine. 2015;12(8):e1001866.

109. Morran MP, Vonberg A, Khadra A, Pietropaolo M. Immunogenetics of type 1 diabetes mellitus. Molecular aspects of medicine. 2015;42:42-60.

110. Morrison NA, Qi JC, Tokita A. Prediction of bone density from vitamin D receptor alleles. Nature. 1994;367.

111. Morrison NA, Yeoman R, Kelly PJ, Eisman JA. Contribution of trans-acting factor alleles to normal physiological variability: vitamin D receptor gene polymorphism and circulating osteocalcin. Proceedings of the National Academy of Sciences. 1992;89(15):6665-9.

112. Motohashi Y, Yamada S, Yanagawa T, Maruyama T, Suzuki R, Niino M, et al. Vitamin D receptor gene polymorphism affects onset pattern of type 1 diabetes. The Journal of Clinical Endocrinology & Metabolism. 2003;88(7):3137-40.

113. Muller K, Bendtzen K. Inhibition of human T lymphocyte proliferation and cytokine production by 1,25-dihydroxyvitamin D3. Differential effects on CD45RA+ and CD45RO+ cells. Autoimmunity. 1992;14.

114. Naidoo Y, Moodley J, Madurai L, Naicker T. Prevalence of Vitamin D deficiency in a multiracial female population in KwaZulu-Natal province, South Africa. South African Family Practice. 2018:1-6.

115. Nejentsev S, Cooper JD, Godfrey L, Howson JM, Rance H, Nutland S, et al. Analysis of the vitamin D receptor gene sequence variants in type 1 diabetes. Diabetes. 2004;53(10):2709-12.

116. Nesby-O'Dell S, Scanlon KS, Cogswell ME, Gillespie C, Hollis BW, Looker AC, et al. Hypovitaminosis D prevalence and determinants among African American

and white women of reproductive age: third National Health and Nutrition Examination Survey, 1988–1994. The American journal of clinical nutrition. 2002;76(1):187-92.

117. Nistico L, Buzzetti R, Pritchard LE. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Hum Mol Genet. 1996;5.
118. Norris JM, Lee H-S, Frederiksen B, Erlund I, Uusitalo U, Yang J, et al. Plasma 25-Hydroxyvitamin D Concentration and Risk of Islet Autoimmunity. Diabetes. 2018;67(1):146-54.
119. Ogunkolade B-W, Boucher BJ, Prahl JM, Bustin SA, Burrin JM, Noonan K, et al. Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to vitamin D status, insulin secretory capacity, and VDR genotype in Bangladeshi Asians. Diabetes. 2002;51(7):2294-300.
120. Ojwang P, Pegoraro R, Rom L, Lanning P. Collagen Io1 and vitamin D receptor gene polymorphisms in South Africa whites, Blacks and Indians. East African medical journal. 2001;78(11):602-7.
121. Omar M, Asmal A. Patterns of diabetes mellitus in young Africans and Indians in Natal. Tropical and geographical medicine. 1984;36(2):133-8.
122. Owerbach D, Gabbay KM. The HOXD8 locus (2q31) is linked to type 1 diabetes. Diabetes. 1995;44.
123. Padgett LE, Broniowska KA, Hansen PA, Corbett JA, Tse HM. The role of reactive oxygen species and proinflammatory cytokines in type 1 diabetes pathogenesis. Annals of the New York Academy of Sciences. 2013;1281(1):16-35.
124. Pani MA, Knapp M, Donner H. Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. Diabetes. 2000;49.
125. Pani MA, Knapp M, Donner H, Braun J, Baur MP, Usadel KH, et al. Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. Diabetes. 2000;49(3):504-7.
126. Panierakis C, Goulielmos G, Mamoulakis D, Petraki E, Papavasiliou E, Galanakis E. Vitamin D receptor gene polymorphisms and susceptibility to type 1 diabetes in Crete, Greece. Clinical immunology (Orlando, Fla). 2009;133(2):276-81.
127. Parikka V, Näntö-Salonen K, Saarinen M, Simell T, Ilonen J, Hyöty H, et al. Early seroconversion and rapidly increasing autoantibody concentrations

predict prepubertal manifestation of type 1 diabetes in children at genetic risk. Diabetologia. 2012;55(7):1926-36.

128. Passini N, Larigan JD, Genovese S, Appella E, Sinigaglia F, Rogge L. The 37/40-kilodalton autoantigen in insulin-dependent diabetes mellitus is the putative tyrosine phosphatase IA-2. Proceedings of the National Academy of Sciences. 1995;92(20):9412-6.
129. Patterson CC, Dahlquist GG, Gyürüs E, Green A, Soltész G, Group ES. Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. The Lancet. 2009;373(9680):2027-33.
130. Pettifor J, Ross F, Solomon L. Seasonal variation in serum 25-hydroxycholecalciferol concentrations in elderly South African patients with fractures of femoral neck. British medical journal. 1978;1(6116):826.
131. Pietropaolo M, Surhigh JM, Nelson PW, Eisenbarth GS. Primer: immunity and autoimmunity. Diabetes. 2008;57(11):2872-82.
132. Pilz S, Iodice S, Zittermann A, Grant WB, Gandini S. Vitamin D status and mortality risk in CKD: a meta-analysis of prospective studies. American Journal of Kidney Diseases. 2011;58(3):374-82.
133. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends in Biochemical Sciences. 2004;29(12):664-73.
134. Pundziute-Lyckå A, Urbonaite B, Ostrauskas R, Zalinkevicius R, Dahlquist GG. Incidence of type 1 diabetes in Lithuanians aged 0–39 years varies by the urban-rural setting, and the time change differs for men and women during 1991–2000. Diabetes Care. 2003;26(3):671-6.
135. Puri S, Marwaha RK, Agarwal N, Tandon N, Agarwal R, Grewal K, et al. Vitamin D status of apparently healthy schoolgirls from two different socioeconomic strata in Delhi: relation to nutrition and lifestyle. British Journal of Nutrition. 2008;99(4):876-82.
136. Raha O, Chowdhury S, Dasgupta S, Raychaudhuri P, Sarkar B, Raju PV, et al. Approaches in type 1 diabetes research: A status report. International journal of diabetes in developing countries. 2009;29(2):85.
137. Ramos-Lopez E, Brück P, Jansen T, Herwig J, Badenhoop K. *CYP2R1* (vitamin D 25-hydroxylase) gene is associated with susceptibility to type 1 diabetes and vitamin D levels in Germans. Diabetes/metabolism research and reviews.

2007;23(8):631-6.

138. Rose K, Penna-Martinez M, Klahold E, Kärger D, Shoghi F, Kahles H, et al. Influence of the vitamin D plasma level and vitamin D-related genetic polymorphisms on the immune status of patients with type 1 diabetes: a pilot study. Clinical & Experimental Immunology. 2013;171(2):171-85.
139. Royal III W, Mia Y, Li H, Naunton K. Peripheral blood regulatory T cell measurements correlate with serum vitamin D levels in patients with multiple sclerosis. Journal of neuroimmunology. 2009;213(1-2):135-41.
140. Sachan A, Agarwal A, Awasthi PK, Gupta R, Das V, Bhatia V. High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India. The American Journal of Clinical Nutrition. 2005;81(5):1060-4.
141. Schmidt KD, Valeri C, Leslie RD. Autoantibodies in Type 1 diabetes. Clinica chimica acta; international journal of clinical chemistry. 2005;354(1-2):35-40.
142. Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, et al. FGF-23 Is a Potent Regulator of Vitamin D Metabolism and Phosphate Homeostasis. Journal of Bone and Mineral Research. 2004;19(3):429-35.
143. Shivaprasad C, Mittal R, Dharmalingam M, Kumar PK. Zinc transporter-8 autoantibodies can replace IA-2 autoantibodies as a serological marker for juvenile onset type 1 diabetes in India. Indian journal of endocrinology and metabolism. 2014;18(3):345.
144. Staples JA, Ponsonby A-L, Lim LL, McMichael AJ. Ecologic analysis of some immune-related disorders, including type 1 diabetes, in Australia: latitude, regional ultraviolet radiation, and disease prevalence. Environmental Health Perspectives. 2003;111(4):518-23.
145. Stene LC, Joner G, Group NCDS. Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study. The American journal of clinical nutrition. 2003;78(6):1128-34.
146. Stenström G, Gottsäter A, Bakhtadze E, Berger B, Sundkvist G. Latent autoimmune diabetes in adults: definition, prevalence, β-cell function, and treatment. Diabetes. 2005;54(suppl 2):S68-S72.
147. Stettler C, Mueller B, Diem P. What you always wanted to know about HbA1c. Schweizerische medizinische Wochenschrift. 2000;130(26):993-1005.

148. Sudhakar D, Kumar EGT, Sarma KV, Srinivasarao PV, Ramalakshmi T, Prasad UV, et al. High prevalence of low dietary calcium, high phytate consumption, and vitamin D deficiency in healthy south Indians. The American Journal of Clinical Nutrition. 2007;85(4):1062-7.
149. Szablewski L. Role of immune system in type 1 diabetes mellitus pathogenesis. International immunopharmacology. 2014;22(1):182-91.
150. Takeyama K-i, Kitanaka S, Kawaguchi Y, Murayama A, Kodera Y, Kato S, et al. Positive and Negative Regulations of the Renal 25-Hydroxyvitamin D3 1α-Hydroxylase Gene by Parathyroid Hormone, Calcitonin, and 1α,25(OH)2D3 in Intact Animals*. Endocrinology. 1999;140(5):2224-31.
151. Tangpricha V, Pearce EN, Chen TC, Holick MF. Vitamin D insufficiency among free-living healthy young adults. The American Journal of Medicine. 2002;112(8):659-62.
152. Thomas HE, McKenzie MD, Angstetra E, Campbell PD, Kay TW. Beta cell apoptosis in diabetes. Apoptosis. 2009;14(12):1389.
153. Thomas MK, Lloyd-Jones DM, Thadhani RI, Shaw AC, Deraska DJ, Kitch BT, et al. Hypovitaminosis D in medical inpatients. New England Journal of Medicine. 1998;338(12):777-83.
154. Thompson SG, Burgess S. Use of allele scores as instrumental variables for Mendelian randomization. International Journal of Epidemiology. 2013;42(4):1134-44.
155. Tizaoui K, Kaabachi W, Hamzaoui A, Hamzaoui K. Contribution of VDR polymorphisms to type 1 diabetes susceptibility: Systematic review of case-control studies and meta-analysis. The Journal of steroid biochemistry and molecular biology. 2014;143:240-9.
156. Turpeinen H, Hermann R, Vaara S, Laine A-P, Simell O, Knip M, et al. Vitamin D receptor polymorphisms: no association with type 1 diabetes in the Finnish population. European journal of endocrinology. 2003;149(6):591-6.
157. Uitterlinden AG, Pols HA, Burger H. A large scale population-based study of the association of vitamin D receptor gene polymorphisms with bone mineral density. J Bone Miner Res. 1996;11.
158. Vanessa O, Asani FF, Jeffery TJ, Saccone DS, Bornman L. Vitamin D receptor gene expression and function in a South African population: Ethnicity, vitamin D and *Fok*I. PLOS one. 2013;8(6):e67663.

159. Vaseghi H, Jadali Z. Th1/Th2 cytokines in Type 1 diabetes: Relation to duration of disease and gender. Indian journal of endocrinology and metabolism. 2016;20(3):312.
160. Vaziri-Sani F, Oak S, Radtke J, Lernmark Å, Lynch K, Agardh C-D, et al. ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. Autoimmunity. 2010;43(8):598-606.
161. Verbeek W, Gombart AF, Shiohara M, Campbell M, Koeffler HP. Vitamin D receptor: no evidence for allele-specific mRNA stability in cells which are heterozygous for the Taq I restriction enzyme polymorphism. Biochemical and biophysical research communications. 1997;238(1):77-80.
162. Verstuyf A, Carmeliet G, Bouillon R, Mathieu C. Vitamin D: a pleiotropic hormone. Kidney international. 2010;78(2):140-5.
163. Viana LV, Gomes MB, Zajdenverg L, Pavin EJ, Azevedo MJ. Interventions to improve patients' compliance with therapies aimed at lowering glycated hemoglobin (HbA1c) in type 1 diabetes: systematic review and meta-analyses of randomized controlled clinical trials of psychological, telecare, and educational interventions. Trials. 2016;17(1):94.
164. Wallberg M, Cooke A. Immune mechanisms in type 1 diabetes. Trends in immunology. 2013;34(12):583-91.
165. Weets I, De Leeuw IH, Du Caju MV, Rooman R, Keymeulen B, Mathieu C, et al. The incidence of type 1 diabetes in the age group 0–39 years has not increased in Antwerp (Belgium) between 1989 and 2000: evidence for earlier disease manifestation. Diabetes Care. 2002;25(5):840-6.
166. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proceedings of the National Academy of Sciences. 2007;104(43):17040-5.
167. White JH. Vitamin D metabolism and signaling in the immune system. Reviews in Endocrine and Metabolic Disorders. 2012;13(1):21-9.
168. Wilmot-Roussel H, Lévy D-J, Carette C, Caillat-Zucman S, Boitard C, Timsit J, et al. Factors associated with the presence of glutamic acid decarboxylase and islet antigen-2 autoantibodies in patients with long-standing type 1 diabetes. Diabetes & metabolism. 2013;39(3):244-9.
169. Winter WE, Schatz DA. Autoimmune Markers in Diabetes. Clinical Chemistry.

2011;57(2):168-75.

170. WITS University. Facts and figures. 2017 [https://www.wits.ac.za/media/wits-university/footer/about-wits/facts-and-figures/documents/Wits-Fact--Figures2017-2018.pdf]
171. World Health Organisation. Blood safety and availability 2017. [https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability]
172. Wong FS, Janeway Jr CA. The role of CD4 vs. CD8 T cells in IDDM. Journal of autoimmunity. 1999;13(3):290-5.
173. Xie Z, Chang C, Zhou Z. Molecular Mechanisms in Autoimmune Type 1 Diabetes: a Critical Review. Clinical Reviews in Allergy & Immunology. 2014;47(2):174-92.
174. Yokota I, Satomura S, Kitamura S, Taki Y, Naito E, Ito M, et al. Association between vitamin D receptor genotype and age of onset in juvenile Japanese patients with type 1 diabetes. Diabetes care. 2002;25(7):1244-.
175. Yoon J-W, Jun H-S. Autoimmune destruction of pancreatic β cells. American journal of therapeutics. 2005;12(6):580-91.
176. Zemunik T, Škrabić V, Boraska V, Diklić D, Terzić IM, Čapkun V, et al. *Fok*I polymorphism, vitamin D receptor, and interleukin-1 receptor haplotypes are associated with type 1 diabetes in the Dalmatian population. The Journal of molecular diagnostics. 2005;7(5):600-4.
177. Zhang J, Li W, Liu J, Wu W, Ouyang H, Zhang Q, et al. Polymorphisms in the vitamin D receptor gene and type 1 diabetes mellitus risk: an update by meta-analysis. Molecular and cellular endocrinology. 2012;355(1):135-42.
178. Zhang L, Eisenbarth GS. Prediction and prevention of Type 1 diabetes mellitus. Journal of diabetes. 2011;3(1):48-57.
179. Zhang Y, Wang X, Liu Y, Qu H, Qu S, Wang W, et al. The GC, *CYP2R1* and DHCR7 genes are associated with vitamin D levels in northeastern Han Chinese children. Swiss medical weekly. 2012;142(2930).
180. Zhao G, Ford ES, Li C, Croft JB. Serum 25-hydroxyvitamin D levels and all-cause and cardiovascular disease mortality among US adults with hypertension: the NHANES linked mortality study. Journal of hypertension. 2012;30(2):284-9.
181. Ziegler A-G, Nepom GT. Prediction and pathogenesis in type 1 diabetes.

Immunity. 2010;32(4):468-78.

182. Zipitis CS, Akobeng AK. Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis. Archives of disease in childhood. 2008;93(6):512-7.

7 Appendices

7.1 Appendix 1: Informed consent and questionnaire

7.1.1 Informed consent

INFORMATION LEAFLET AND INFORMED CONSENT FORM FOR ADULTS FOR CLINICAL STUDY / INTERVENTION RESEARCH

1. STUDY TITLE

The role of vitamin D in the aetiology of type 1 diabetes mellitus (T1D) in the South African Indian and White populations.

2. THE NATURE AND PURPOSE OF THIS STUDY

Hello. My name is _____ , and I am doing a research study on diabetes and the effect that vitamin D might have in causing this disease. I am a student at WITS doing a research study for my Masters degree. The aim of this study is to evaluate the role genes and antibodies play in causing diabetes.

Diabetes affects different age groups:

Babies Kids Young Adults Older Adults

Some diabetics are very thin and some are overweight. Why diabetes should affect these different types of people is not very clear.

Genes are present in every cell of your body and they are responsible for the differences we see between people (like hair colour for example). Different genes may also explain why some people get diabetes and why some people don't. Antibodies are molecules that your body makes to protect itself against strange proteins in your body (your body makes antibodies against germs for example). Sometimes the body makes antibodies against itself. It can make against antibodies against insulin for example and that may cause diabetes.

We want to study the genes and antibodies in people with diabetes. We are especially interested in vitamin D as studies have shown that that low levels of vitamin D can increase a person's risk of developing diabetes. We would also like to look at the genes that are linked to vitamin D (the genes to make vitamin D and involved in the transport of vitamin D in the body) as these have also been shown to be linked to the risk of getting diabetes.

This information leaflet is to help you decide if you would like to participate in our study. You should fully understand what is involved before you agree to take part in this study.

If you have any questions, please don't hesitate to ask me.

3. EXPLANATION OF PROCEDURES TO BE FOLLOWED

Should you agree to volunteer, the study involves answering some questions with regard to your diabetes, sun exposure, weight, height, hip and waist measurements and blood drawn. This will all be done on the same day as you are visiting the clinic. Three blood samples (5ml each) will be taken and some blood will be analysed and some blood samples will be stored for testing at a later stage. Any tests done on these blood samples will only be related to diabetes.

4. RISK AND DISCOMFORT INVOLVED

The only risk and discomfort involved is the taking of blood from a vein and the measurement of your waist and hips.

5. POSSIBLE BENEFITS OF THIS STUDY

The findings of a study such as this will not benefit you personally but will lead to a better understanding of how diabetes and possibly its complications is caused and may lead to new treatments in future. For those who do not have diabetes we will be testing your glucose so that if you have high blood glucose (diabetes) when we test you, you will be referred for the right treatment.

6. If you do not want to participate in this study, you will still receive standard treatment for your illness.

7. You may withdraw from this study at any time.

8. INFORMATION

If you have any questions concerning this study, you should contact: Dr C Padoa, Tel : 011 489 8514.

9. CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that patients remain unidentifiable. All samples will be given a unique study number and any links to the participant will be kept in a separate file stored securely in Dr Padoa's office in the department of Chemical Pathology. This information will only be available to Dr Padoa.

CONSENT TO PARTICIPATE IN THIS STUDY

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

....

Patient's name Patient's signature

Hospital number:.....

Telephone number of self or best possible contact:.....

Date.....

....

Investigator's name Investigator's signature

Date.....

VERBAL PATIENT INFORMED CONSENT

(applicable when patients cannot read or write)

I, the undersigned, Dr ....., have read and have explained fully to the patient, named ..... and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his/her illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardising his/her further treatment.

I hereby certify that the patient has agreed to participate in this trial.

....

Patient's name Witness's signature

Date.....

....

Investigator's Name Investigator's Signature

7.1.2 Questionnaire

Date.....

Questionnaire:

Demographics

Study number
Date of visit (dd/mm/yyyy):
Biogram
Age:
Gender: M / F
Race:
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:
Urine dipstick (from file): *Glucose:* *Ketones:* *Protein:* *Blood:*
Blood pressure (mmHg):
Weight (kg):
Height (cm):
Waist circumference (cm):
Hip circumference (cm):
Acanthosis Nigricans: Y / N / U Where:
Clinical judgement on type of DM: 1 / 2 DM year of diagnosis:
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:

4 of 5

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

1. Please circle which best describes your main occupation:

Mainly indoors
Half indoors and half out doors
Mainly outdoors

7.2 Appendix 2: Ethics Certificates

7.2.1 Wits Human Ethics Committee certificate

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms Nompumelelo Malaza

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180536

NAME: (Principal Investigator)	Ms Nompumelelo Malaza
DEPARTMENT:	Chemical Pathology The Department of Chemical Pathology Wits Medical School Laboratory 3Q07
PROJECT TITLE:	The role of vitamin D in the aetiology of type 1 diabetes mellitus (T1D) in the South African Indian and White populations
DATE CONSIDERED:	25/05/2018
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Carolyn Padoa
APPROVED BY:	Professor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	06/08/2018

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 **May** and will therefore be due in the month of **May** 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

7.2.2 SANBS Ethics Committee certificate

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**

7.3 Appendix 3: PCR gels

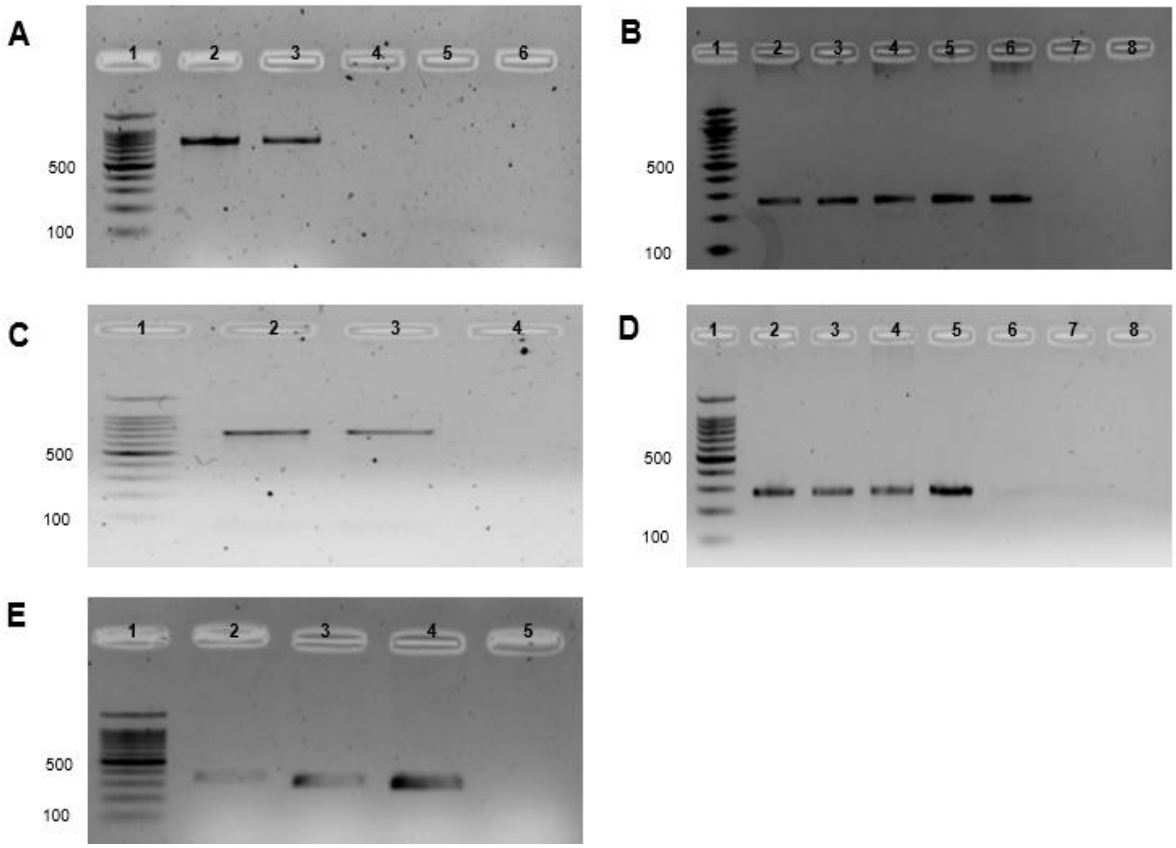

Figure 7.1: PCR products *VDR* ((A) *Bsm*I, (B) *Fok*I, (C) *Apa*I/ *Taq*I and vitamin D metabolising enzyme gene((D) *CYP2R1* and (E) *CYP27B1*) run on a 1% agarose gel

A: Lane 1: 100 bp ladder, lane 2 and 3: 822 bp PCR product, lane 6: negative control

B : Lane 1: 100 bp ladder, lane 2-6: 267 bp PCR product, lane 8: negative control

C: Lane 1: 100 bp ladder, lane 2 and 3: 754 bp PCR product, lane 4: negative controls

D: Lane 1: 100 bp ladder, lane 2-5: 287 bp PCR product, lane 8: negative control

E: Lane 1: 100 bp ladder, lane 2-4: 317 bp PCR product, lane 5: negative control

7.4 Appendix 4: Chromatograms

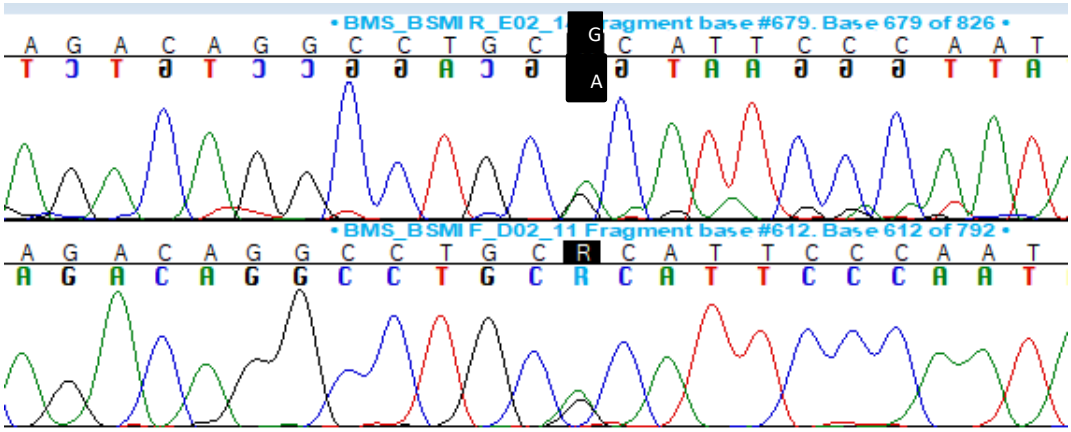

Figure 7.2: Chromatograms showing the portion of the *VDR* gene containing the *Bsm*I polymorphism, from individuals genotyped as heterozygous GA for the *Bsm*I polymorphism through PCR-RFLP.

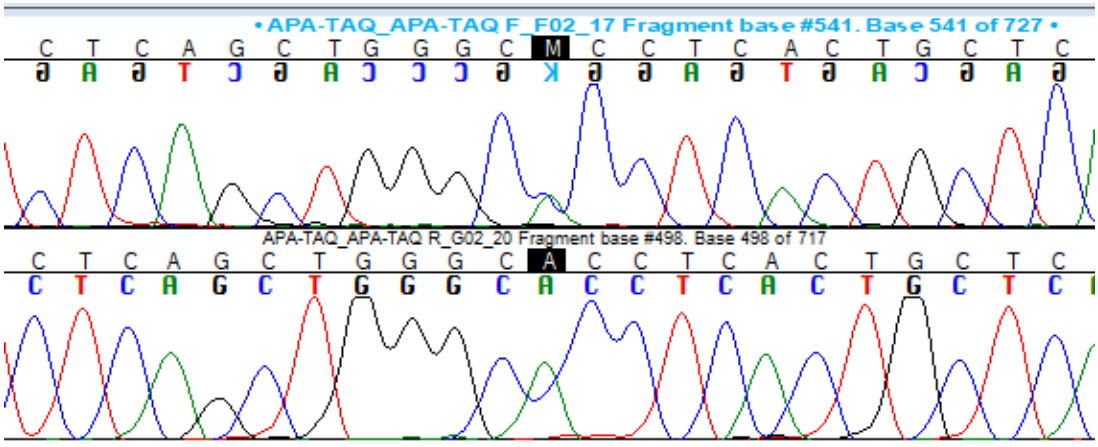

Figure 7.3: Chromatograms showing the portion of the *VDR* gene containing the *Apa*I polymorphism, from individuals genotyped as heterozygous CA (top) and homozygous AA (bottom) for the *Apa*I polymorphism through PCR-RFLP.

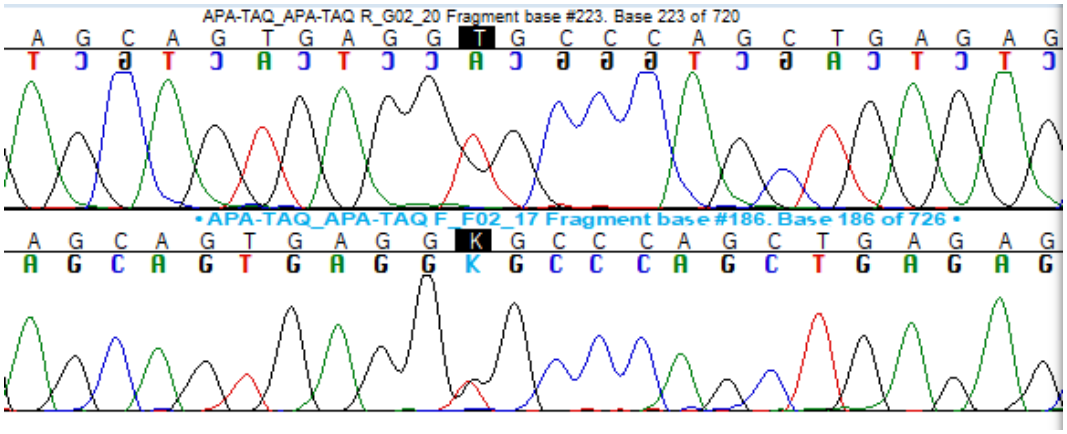

Figure 7.4: Chromatograms showing the portion of the *VDR* gene containing the *Taq*I polymorphism, from individuals genotyped as homozygous TT (top) and heterozygous GT (bottom) for the *Taq*I polymorphism through PCR-RFLP.

7.5 Appendix 5: Real time PCR amplification graphs and melt curves

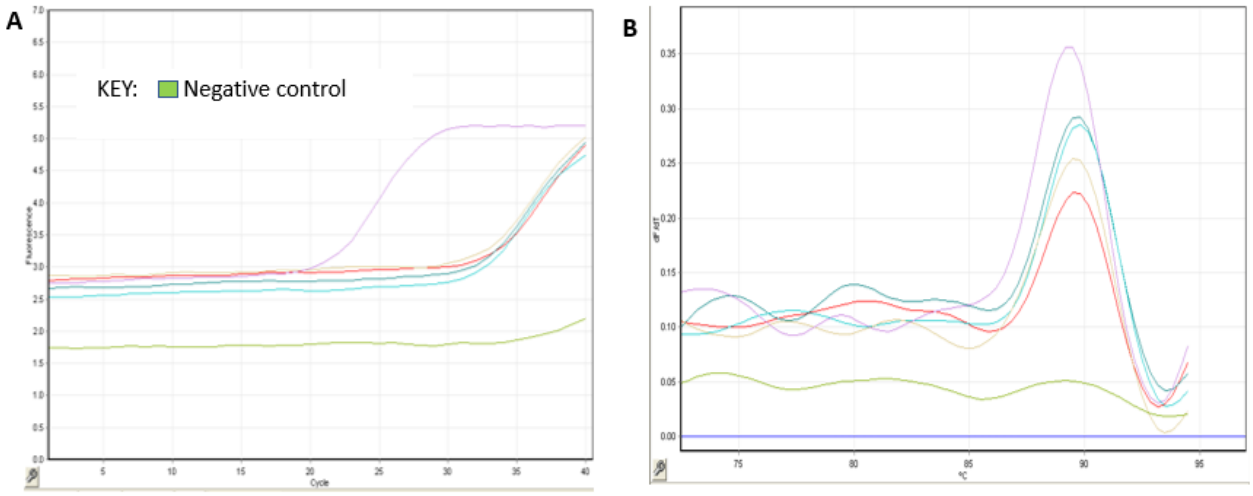

Figure 7.5: Amplification (A) and melt (B) curves for quantification of the *VDR* gene

The negative control is represented by the green colour and participant samples by the remaining colours

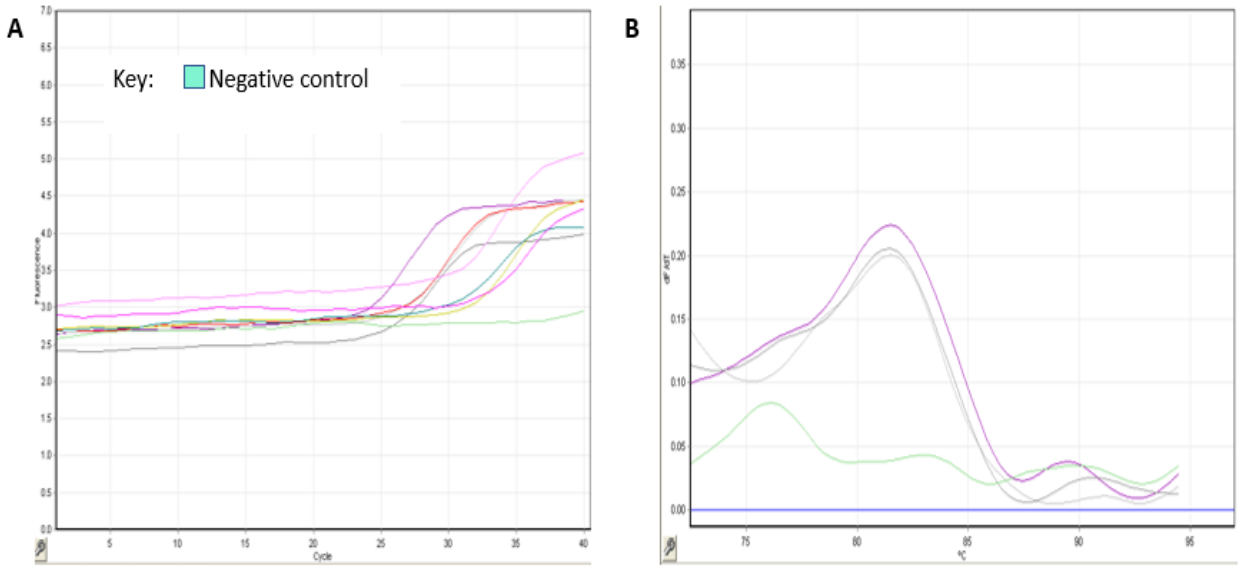

Figure 7.6: Amplification (A) and melt (B) curves for quantification of the *β-actin* gene

The negative control is represented by the green colour and participant samples by the remaining colours

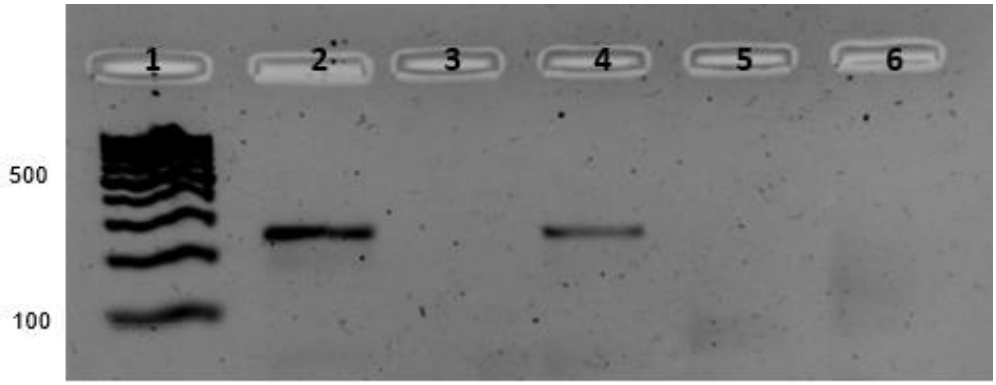

Figure 7.7: Amplification products of the *VDR* and *β-actin* genes used for relative quantification run on a 1% gel showing no contamination but primer dimer amplification in the negative controls.

Lane 1: 100 bp ladder; lane 2: *β-actin* PCR product; lane 3: empty; lane 4: *VDR* gene PCR product; lane 5: *β-actin* negative control, lane 6: VDR negative control

7.6 Appendix 6: Turn-it-in report

670320:Mpumi_dissertation_for_turn_it_in.docx

ORIGINALITY REPORT

3%	2%	2%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	epdf.tips Internet Source	2%
2	Vitamin D, 2010. Publication	1%
3	Roberd M. Bostick. "Calcium and Vitamin D", Genetics of Colorectal Cancer, 2009 Publication	1%

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		