When too much sun is never enough: Association of the VDR gene polymorphisms with insulin resistance

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A thesis is submitted to
Auckland University of Technology
in partial fulfilment of the requirements for the degree
of Master of Applied Science (MAppSc)

2010

School of Applied Sciences

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a University or other institution of higher learning.

Reema Jain

Acknowledgements

This work was undertaken during the year 2009-2010 at the Faculty of Health and Environmental Sciences of Auckland University of Technology (AUT). My work would be incomplete without mentioning the names of the people, who with their utmost support and inspiration made this project a success.

To begin with, I owe my deepest gratitude to Dr. Colleen Higgins, my primary supervisor and mentor who supported me throughout my thesis and allowed me to work in my own way. Her encouragement and efforts have always kept me going. Her guidance and supervision in the laboratory were of great help.

I am very much grateful to Dr. Donald Love (University of Auckland), my secondary supervisor, who was there to guide me right from the day of commencement of my work, and also with helping me in editing my thesis.

I am eternally greatful to the contributions made by Dr. Pamela von Hurst and Dr. Jane Coad of Massey University and providing me with this opportunity to continue working for their Surya project.

My very sincere thanks to Welma Stonehouse who provided me with great help in processing the data, understanding statistics, in understanding the measurements that were to be taken in the experiments conducted and for the interpretation of results.

The Technicians present in the laboratories of AUT have also done a great deed with aiding me in this project. The efficient running of the laboratory is much more a testament to their sincere efforts than mine.

I would also like to thank the Massey University Human Ethics Committee (Southern A), Ref No.06/67, for the confidence shown in me by the trustees and also for approving the Surya Study and this project. Without their faith, the completion of this project would not have been realised.

I am indebted to my family and friends and offer my deepest gratitude to them for their indefatigable love and blessings. This thesis would not have been possible without their support.

I would like to conclude with a vote of thanks to all the other people whose names cannot be given in a very short space for their patronage.

Abstract

The metabolism of vitamin D commences with exposure of the skin to sunlight. The growing recognition of its role in insulin resistance, autoimmune disorders, infections, cancer, as well as the health of cells that influence physical and mental function have profound implications on how we define vitamin D requirements and why we should care whether they are met or not. Most of the actions of vitamin D are mediated by the vitamin D receptor (VDR), a protein whose gene sequence can vary, giving rise to polymorphic forms which are potent enough to affect the binding capacity of this protein to vitamin D. Some of these polymorphic forms of *VDR* gene may be associated with reduced effectiveness of vitamin D and hence predispose individuals to diseases such as type 2 diabetes and insulin resistance. An earlier study, the Surya Study, looked at the responsiveness of the South-Asian women living in Auckland to vitamin D. The research described here is an extension of this study and its focus was to identify the associations/linkages between certain polymorphic forms of the *VDR* gene and the disease conditions and intervention responsiveness in the same women.

The first objective was to compare two well known techniques for genotyping single nucleotide polymorphisms (SNPs) of the *VDR* gene at the 3' end, namely *Bsm*I, *Apa*I and *Taq*I: the newer real-time polymerase chain reaction (qPCR) and the traditional restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) techniques. This comparison was performed to evaluate alternative methods for genotyping which consumed less time than RFLP-PCR. When the presence of each polymorphism by both the techniques was compared in this cohort of South-Asian women, it was found that RFLP-PCR proved to be a more reliable technique than qPCR for genotyping the *VDR* gene.

Another objective of this project was to investigate the prevalence of the above three polymorphisms along with Cdx-2 and *Fok*I SNPs which are present at the 5' end of the *VDR* gene, in the population under study and their possible association with phenotypes such as vitamin D responsiveness and insulin resistance. These women were screened and biochemical data was collected during the earlier Surya Study. Of these, eighty-one women were then selected for intervention based on them having high insulin resistance (HOMA-

IR>1.93) and serum 25(OH)D<50 nmol/L. Out of these eighty-one women, forty-two were given vitamin D supplement and thirty-nine were given a placebo for six months. Baseline and endpoint measurements included insulin resistance (HOMA-IR), insulin sensitivity (HOMA2%S) etc. How each individual responded to treatment in the intervention group was analysed in the context of the polymorphisms that they had. An association of insulin resistance with *Bsm*I, *Apa*I and *Taq*I SNPs was observed in this cohort of 239 women. The response to insulin resistance in the vitamin D supplemented group significantly differed for *Fok*I genotype compared to other genotypes. This explained why certain women responded to treatment better than the others.

When the frequencies of the genotypes of these five SNPs of the *VDR* gene were compared to other studies of different ethnicities, the results of this study were consistent with few studies but contradictory to others. The possible reasons for these differences could be because of small sample size and different ethnicities under study due to which the frequency of alleles and hence the genotypes differed.

Chapter 1

General Introduction

1.1. What is vitamin D?

The sun is the source of vitamin D, an essential fat-soluble vitamin whose production begins when the skin is exposed to ultra violet-β (UVB) radiation. Vitamin D achieved prominence in 1920 when it was discovered as an important vitamin required for calcium homeostasis (Norman, 2006).

In 1933, the biological importance of vitamin D and the necessity for daily exposure to the sun's ultraviolet rays was recognised in the prevention of the disease rickets in children (Bakwin & Bakwin, 1933). This disease is the consequence of a decrease in bone mineralization that leads to skeletal malformation (Plum & DeLuca, 2009). In adults, osteomalacia is the result of vitamin D deficiency where bones are easily fractured. These diseases became prevalent during the Industrial Revolution when smoke and urbanization blocked the sunlight reaching the earth's surface (Plum & DeLuca, 2009). Eventually, the importance of vitamin D was established with its classical role in bone and calcium homeostasis.

1.2. Synthesis of vitamin D

Vitamin D exists in two forms: vitamin D_3 or cholecalciferol, and vitamin D_2 or ergocalciferol (Figure 1.1). Vitamin D_3 is produced in the skin by the action of UVB (280-320nm) on 7-dehydrocholesterol (7-DHC) to produce pre-vitamin D_3. This occurs after 30 minutes of exposure (Figure 1.1). This photochemical conversion in the skin proceeds rapidly. Body heat then causes pre-vitamin D_3 to undergo a thermal isomerisation to vitamin D_3 (Lehmann, 2009; Zittermann, 2003). In plants, the action of ultraviolet radiation (UVR) on ergosterol produces pre-vitamin D_2 which is finally converted to vitamin D_2 due to heat (Bikle, 2009a).

Vitamin D_2 and vitamin D_3 are similar with respect to their mechanism of action at the cellular level, in particular gene activation. Therefore, references to vitamin D or vitamin D metabolites indicate both vitamin D_2 and vitamin D_3 unless specified with a subscript (Bikle, 2009b). To be biologically active, vitamin D must be transported through the blood

bound to either vitamin D binding protein (DBP) or albumin to the liver where it is converted to 25(OH)D by a number of cytochrome P450 enzymes (Plum & DeLuca, 2009). Vitamin D_3 binds DBP more efficiently than vitamin D_2 with the result that single doses of vitamin D_3 are likely to increase circulating 25(OH)D levels to a greater degree compared to vitamin D_2 (Armas, Hollis, & Heaney, 2004). However, if administered regularly in equal doses, their blood levels become comparable (Holick et al., 2008). To be fully active, 25(OH)D must be transported to other cell types where it is converted to the hormonally active form calcitriol $1,25(OH)_2D$, *via* CYP27B1, a mitochondrial P450 enzyme (Bikle, 2009b).

Initially, it was thought that CYP27B1 was found only in renal tissues. However, later it was discovered that many non renal tissues such as immune cells, keratinocytes, bone, placenta, prostate, parathyroid cells, transformed B-cells, cancer cells and several other tissues contain this enzyme. The variety of tissues is thought to be responsible for local production of CYP27B1 protein, and tissue-specific response to vitamin D. Of these, kidney is the major site of $1,25(OH)_2D$ production. From the kidney, $1,25(OH)_2D$ is released to the blood stream and circulated around the body to the target sites (Hewison et al., 2007).

Production of $1,25(OH)_2D$ by CYP27B1 is controlled by the levels of calcium, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF 23) and by $1,25(OH)_2D$ itself through a feedback mechanism. Calcium along with PTH stimulate, while FGF 23 inhibits, $1,25(OH)_2D$ production (Bikle, 2009a). $1,25(OH)_2D$ down regulates its own levels by inducing CYP24 (25-OHase) (Nishimura et al., 1994), another mitochondrial P450 enzyme that degrades both $1,25(OH)_2D$ and 25(OH)D (Zierold, Darwish, & DeLuca, 1995).

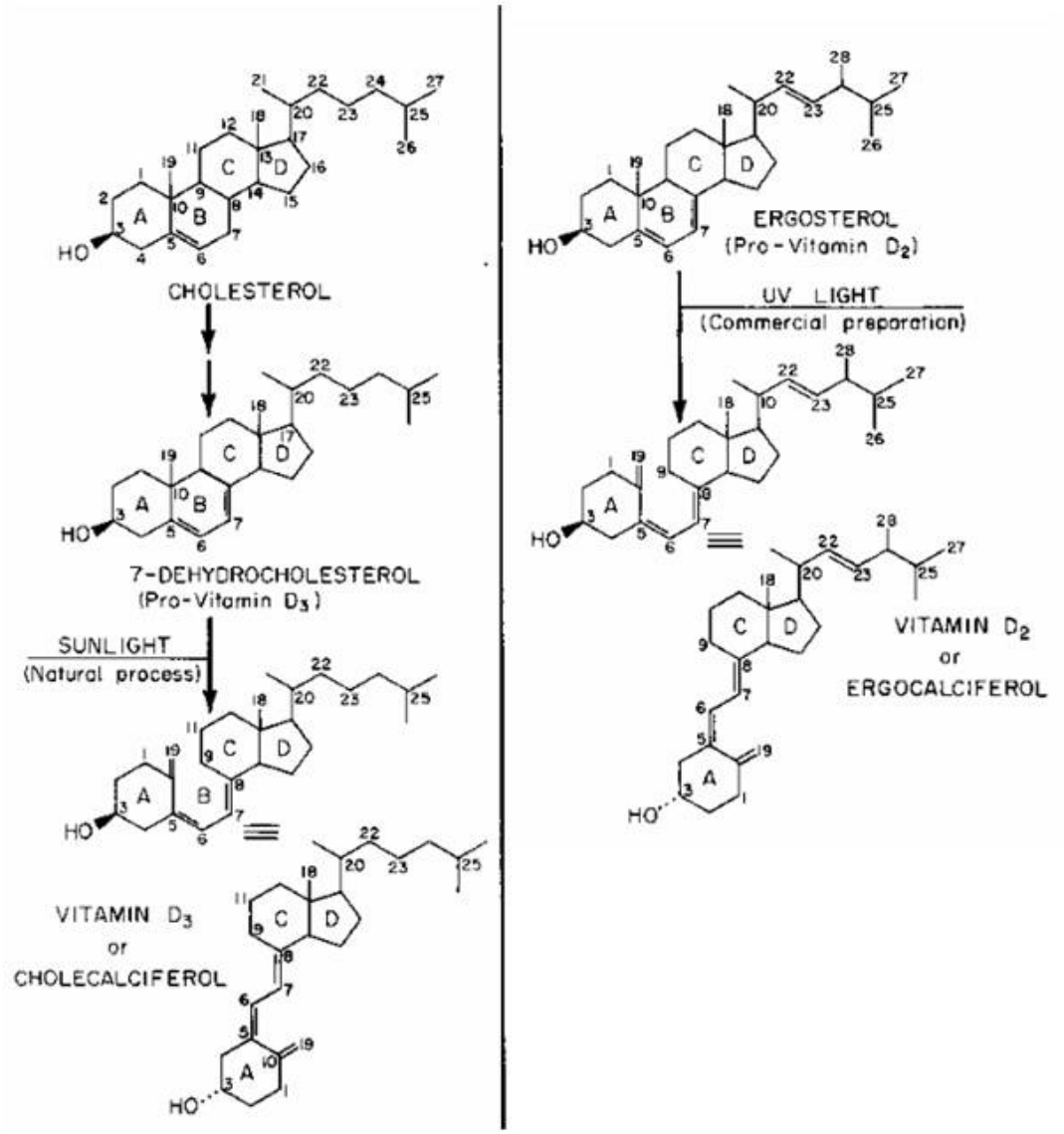


Figure 1.1. A schematic representation of vitamin D_3 and vitamin D_2 synthesis from cholesterol and ergosterol, respectively.

From "Chemistry of Vitamin D (1999)". Retrieved October 28, 2009, from http://vitamind.ucr.edu/chem.html.

Vitamin D is taken into cells following binding to the vitamin D receptor (VDR). At the cellular level, the biological activities of both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ are comparable with respect to this binding (Bikle, 2010). VDR, a nuclear hormone receptor that mediates most of the functions of vitamin D is found in most tissues of the body (Bikle, 2010). Thus, it is suspected that due to the presence of VDR and CYP27B1 in most

tissues, $1,25(OH)_2D$ not only exerts its actions in the classical tissues such as those involved in calcium metabolism (gut, bone and kidney), but also in other tissues such as neurons, placenta, ovarian cells and pituitary cells (Bikle, 2010).

There are various factors that contribute to vitamin D formation such as UVR intensity, latitude, season, age and skin pigmentation. Generally, in winter and higher latitudes, healthy young adults have lower levels of 25(OH)D compared to lower latitudes and summer (Lamberg-Allardt, Outila, Kärkkäinen, Rita, & Valsta, 2001). Even children engaged in outdoor activities with frequent exposure to sunlight will have lower 25(OH)D levels at higher latitudes (Davies, Bates, Cole, Prentice, & Clarke, 1999). The main reason is that vitamin D synthesis largely depends on skin synthesis which in turn depends on sun exposure, which is fairly low in high latitudes and in winter (Zittermann, 2003).

In general, elderly subjects have lower levels of 25(OH)D compared to young adults as they indulge in fewer outdoor activities, wear clothes with long sleeves and have a marked decrease in the capacity of their skin to synthesize vitamin D (Wielen et al., 1995). Another factor is the skin pigment, melanin. As for clothing and sunscreen, melanin reduces the efficiency of UVB- mediated vitamin D formation as it blocks UVR reaching 7-DHC, necessitating increasing the exposure time required to reach optimal levels of vitamin D in the body (Lawson, Thomas, & Hardiman, 1999). Therefore, those with lighter skin are better able to synthesize vitamin D compared to those who are dark-skinned, thereby avoiding the symptoms associated with vitamin D deficiency. However, the higher concentration of melanin in darker skin at low latitudes prevents the harmful effects of UVR such as the cutaneous damage and loss of low concentrated valuable micronutrients including folate (Jablonski & Chaplin, 2000).

1.3. Measurement of vitamin D levels

A candidate reference measurement to assess the levels of vitamin D in the body has been debated regarding using $1,25(OH)_2D$ as an indicator of vitamin D levels in the body. Even though $1,25(OH)_2D$ is the biologically active form, unlike 25(OH)D, its level is maintained

in the blood until the extremes of vitamin D deficiency are manifest due to secondary hyperparathyroidism. Hence, it is not a good indicator for assessing vitamin D deficiency at the initial stages (Bikle, 2010). A circulating blood level of 25(OH)D of <20 ng/ml (<50 nmol/L) has been defined as vitamin D deficiency, 21-29 ng/ml (52.5-72.5 nmol/L) vitamin D insufficiency and >30 ng/ml (>75 nmol/L) vitamin D sufficiency (Holick, 2008). Therefore, levels of 25(OH)D >30ng/ml is recommended for normal functioning of the body (Holick, 2008). The required levels of vitamin D can be achieved through diet, supplements and sun exposure.

Vitamin D_2 from the diet, is ingested as a a prohormone from dietary sources such as oily fish, salmon, mackerel and herring, cod liver oil, tuna and shark as well as sun-dried mushrooms (Holick, 2008). Very few countries have foods that are fortified with vitamin D. For example, in the US, a few milk products are fortified with vitamin D. However, the amount of fortification at 100 IU (1 IU is 0.025 μg) per serving is not enough to prevent vitamin D deficiency in children or adults as it only increases the blood level of the major circulating form of vitamin D, 25(OH)D by 1ng/ml (Holick, 2008). Apart from food fortification, vitamin D supplements can also be recommended as a dietary supplement to increase vitamin D levels in the body. It is recommended that perhaps all adults need approximately 800-1000 IU daily. Alternatively, a single weekly dose of 5000 IU should be able to increase 25(OH)D levels to normal range (Utiger, 1998) . However, since there is individual variation in terms of vitamin D production by skin and its subsequent metabolism, it is impractical to make a 'one size fits all' recommendation with regards to oral supplementation (Bikle, 2010; Holick, 2008). One of the classic examples is the Surya Study by Hurst et al. (2008). This was a randomised controlled trial where forty-two individuals were given 4000 IU of vitamin D_3 per day and thirty-nine a placebo for six months. These individuals had 25(OH)D <50 nmol/L. At the end of six months, when their vitamin D levels were assessed, it increased slightly for few individuals only and therefore, larger dose was recommended for a sufficient length of time. This shows how individual variation in vitamin D status makes it impractical to recommend a dose for all. However, one of the questions still left unanswered is whether or not oral supplements of vitamin D have the same effect as endogenous production by skin on sun exposure (Bikle, 2010). This

question becomes important when designing vitamin D treatment strategies for individuals with varying amounts of vitamin D.

A few cases have been published revealing that vitamin D is similar to vitamin A in toxicity and could induce severe hypercalcemia (increased circulating calcium levels), nephrocalcinosis (deposition of calcium in renal tissues) and cardiovascular disorders. In all cases of vitamin D toxicity, levels >200 nmol/L of 25(OH)D have been clearly observed (Jacobus et al., 1992; Markestad, 1987). Levels as high as 1000 nmol/L of 25(OH)D have also been detected (Cavalier, Delanaye, Chapelle & Souberbielle, 2009). These clinical symptoms of intoxication need enormous amounts of vitamin D to be ingested. Intoxication of vitamin D was described in British infants during the late 1940s after they were fed with dried milk powder enriched with vitamin D in addition to the daily recommendation of 17.5-20 µg of vitamin D supplements (Chesney, 1989). However, there have been no reports of toxicity with excessive exposure to sunlight (Zittermann, 2003). UVB exposure until sunburn does not result in toxicity as prolonged exposure results in the formation of non-biologically active products (Zittermann, 2003).

1.4. Metabolic functions of vitamin D

1.4.1. Skeletal mineralisation

The association of vitamin D with bone mineralisation led to the understanding that the classical function of $1,25(OH)_2D$ is to increase the calcium and phosphorus absorption from the intestine for skeletal mineralisation (Wong, Norman, Reddy, & Coburn, 1972). The body maintains its physiological range of extracellular ionised calcium to maintain cellular signal transduction and most metabolic functions (Holick, 2008). This is done by the conversion of 25(OH)D to $1,25(OH)_2D$, which then acts on the intestine to increase absorption of calcium. If the circulating calcium level is still inadequate, $1,25(OH)_2D$ increases absorption of calcium and phosphorus from the kidney and acts on bone osteoclasts to mobilise the calcium from the skeleton into the blood stream (Figure 2.1) (Holick, 2008; Palomer, Gonzalez-Clemente, Blanco-Vaca & Mauricio, 2008). Only when

the body's calcium level is adequate, will 25(OH)D be converted by other tissues to $1,25(OH)_2D$.

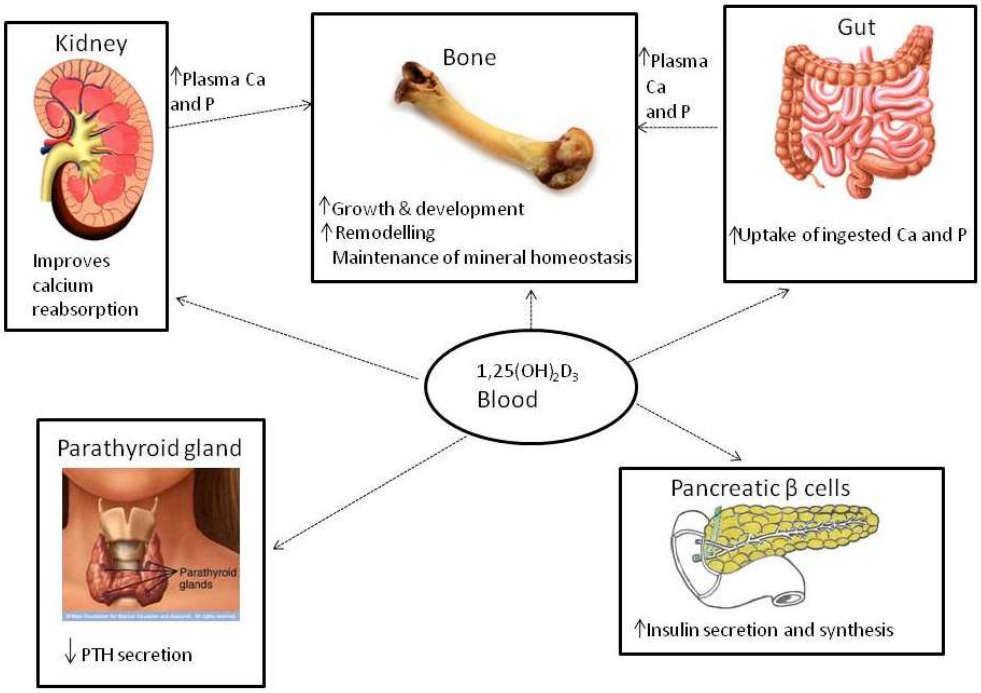


Figure 1.2. The main targets of vitamin D within the body-kidney, bone, gut, parathyroid gland and pancreatic β cells. PTH-parathyroid hormone; Ca-Calcium; P-phosphorus.

Modified from "Role of vitamin D in the pathogenesis of type 2 diabetes mellitus," by X. Palomer, J. M. Gonzalez-Clemente, F. Blanco-Vaca and D. Mauricio, 2008, *Diabetes, Obesity and Metabolism*, *10*, p.187.

1.4.2. Regulation of hormone secretion

Vitamin D regulates the production and secretion of various hormones that in turn either inhibit or stimulate $1,25(OH)_2D$ production. Figures 1.2 and 1.3 show the regulation of these hormones which in some cases inhibit production of $1,25(OH)_2D$ in kidney, which is the main source of $1,25(OH)_2D$ production. These are described below.

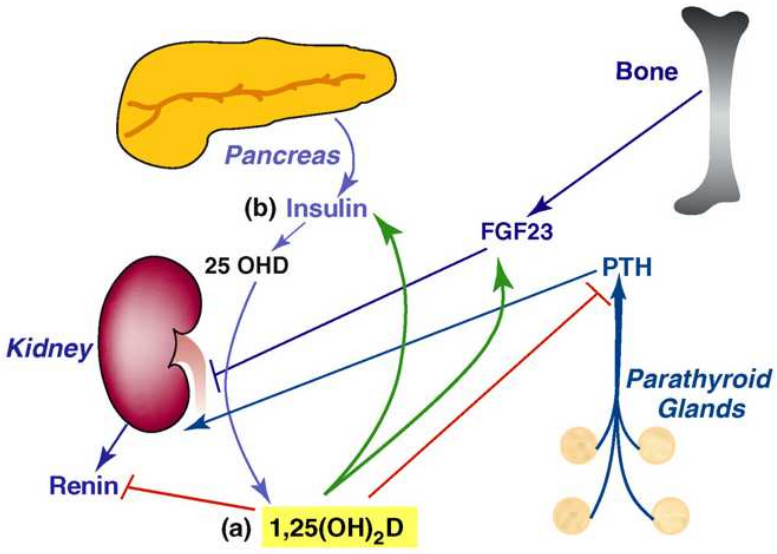


Figure 1.3. Hormonal regulation of and by $1,25(OH)_2D$. (a) Green arrows show stimulation of insulin and FGF 23 production by $1,25(OH)_2D$ whereas red lines show inhibition of production of PTH and renin. PTH and FGF 23 are major vitamin D regulators, the former stimulates and the latter inhibits renal production of $1,25(OH)_2D$. (b) Insulin can also stimulate conversion of 25(OH)D to $1,25(OH)_2D$, perhaps through insulin-like growth factor (IGF) receptor. PTH- Parathyroid hormone; FGF 23- Fibroblast growth factor 23.

From "Vitamin D: newly discovered actions require reconsideration of physiologic requirements," by Daniel D. Bikle 2010, *Trends in Endocrinology and Metabolism*, *n.d*., p.4. Copyright©2010 by Elsevier Ltd.

1.4.2.1. Parathyroid hormone (PTH)

$1,25(OH)_2D$ inhibits the synthesis of PTH and prevents cell proliferation in the parathyroid gland (Demay, Kiernan, DeLuca, & Kronenberg, 1992) (Figure 1.3). In contrast, PTH stimulates the renal conversion of 25(OH)D to $1,25(OH)_2D$ under various physiological conditions such as when calcium levels are low (Wong et al., 1972). Under low calcium levels, the calcium receptors (CaSR) in the parathyroid gland become activated and stimulate the production and secretion of PTH. The latter is secreted under hypocalcemia, but not when calcium levels are adequate (Canaff & Hendy, 2002). PTH continues to stimulate the conversion of 25(OH)D to $1,25(OH)_2D$ by 25(OH)D-1-hydroxylase until calcium levels are normal. This stimulation increases the circulating levels of $1,25(OH)_2D$ which, together with PTH, acts on the kidney and intestine to increase calcium absorption (Martin & Deluca, 1969). When calcium levels return to normal, $1,25(OH)_2D$ together with calcium inhibit PTH secretion through their specific receptors VDR and CaSR, respectively

(Plum & DeLuca, 2009). This is an important feedback loop by which PTH levels are controlled by $1,25(OH)_2D$. These actions of $1,25(OH)_2D$ are clinically exploited where patients with vitamin D deficiency and renal failure have high levels of PTH resulting in secondary hyperparathyroidism (Martin & González, 2004). Patients with diseased kidneys are unable to synthesise $1,25(OH)_2D$ resulting in very low levels of $1,25(OH)_2D$ and high levels of PTH. Vitamin D deficiency may also result in increased bone turnover, leading to rickets in children, osteomalacia and disturbed muscle metabolism in adults (Palomer et al., 2008). Therefore, treatment of osteomalacia and secondary hyperparathyroidism with high doses of calcium and vitamin D are very effective (Amin, Wall, & Cooke, 2007).

1.4.2.2. Fibroblast Growth Factor 23 (FGF 23)

FGF 23 is primarily produced by osteoblasts and osteocytes and is stimulated by $1,25(OH)_2D$ (Kolek et al., 2005). Unlike PTH, it inhibits the production of $1,25(OH)_2D$ by the kidney (Figure 1.3) (Shimada, Hasegawa et al., 2004; Shimada, Kakitani et al., 2004). This feedback loop, like that of PTH, maintains levels of FGF 23 in the blood stream and therefore, FGF 23 overproduction may lead to vitamin D deficiency (Bikle, 2010). FGF 23 regulates phosphate and 25(OH)D levels in the blood. It regulates phosphate metabolism and like $1,25(OH)_2D$, phosphorus also regulates FGF 23 production (Shimada, Kakitani et al., 2004). Hence, levels of FGF 23 are tightly regulated in the blood stream with the result that deficiency of FGF 23 causes severe hyperphosphatemia (Shimada, Hasegawa et al., 2004).

1.4.2.3. Renin

The juxtaglomerular cells of the kidney produce renin that converts angiotensinogen to angiotensin I, that is subsequently converted to angiotensin II, which is a major regulator of hypertension and cardiac hypertrophy (Bikle, 2010). $1,25(OH)_2D$ inhibits the production of renin by the kidneys (Figure 1.3). Thus, increased levels of renin during vitamin D

deficiency can explain the inverse correlation between vitamin D deficiency and hypertension and cardiac disease (Li et al., 2002; Xiang et al., 2005).

1.4.2.4. Insulin

1.4.2.4a. β cell function

Vitamin D has also been shown to regulate levels of insulin and to maintain normal glucose tolerance (Boucher, Mannan, Noonan, Hales, & Evans, 1995), such that deficiency of vitamin D leads to an impairment of insulin secretion, and diabetes becomes irreversible (Boucher et al., 1995). A study by Scragg et al. (1995) showed that diabetes and impaired glucose tolerance (IGT) are associated with a low serum concentration of $25(OH)D_3$ in New Zealand Polynesians, which may be partly responsible for the increased incidence of diabetes/IGT in this population compared to Europeans. There is ample evidence to show that vitamin D induces the synthesis and secretion of insulin by β cells in the pancreas (Figure 1.2, 1.3). Firstly, the presence of VDR in β cells may induce transcription of insulin through vitamin D action (Johnson, Grande, Roche, & Kumar, 1994). Secondly, there have been numerous reports on the improvement of insulin secretion by vitamin D supplementation in patients with mild type 2 diabetes mellitus and vitamin D deficiency, thereby improving their glucose tolerance (Borissova, Tankova, Kirilov, Dakovska, & Kovacheva, 2003; Ismail & Namala, 2000; Tanaka, Seino, & Ishida, 1986). This is followed by an increase in calcium levels and a reduction in free fatty acids (Tanaka et al., 1986). However, conflicting results have been reported where vitamin D supplementation in three vitamin D deficient individuals of South-Asian origin had increased insulin resistance and imbalanced glycemic control (Taylor & Wise, 1998). The contradictory results of this study could be explained by the extremely small sample size (n=3). Also, the study reported an increase in fasting insulin levels after three months following vitamin D supplementation, but used a Fasting Insulin Resistance Index (FIRI) to measure insulin resistance, which has not been fully validated (Taylor & Wise, 1998). Therefore, the results were highly biased because of small sample size and wrong measurement for insulin resistance.

There could be several pathways through which vitamin D promotes the secretion of insulin (Palomer et al., 2008). Vitamin D promotes uptake of calcium by β cells *via* non-selective voltage-dependent calcium channels, thus increasing its intracellular calcium levels (Sergeev & Rhoten, 1995). Increased calcium levels stimulate the cleavage of proinsulin to insulin by endopeptidase (Chiu, Chu, Go, & Saad, 2004). Moreover, calcium is also necessary for the exocytosis of insulin from β cells. Vitamin D and serum phosphorus also activate protein biosynthesis in pancreatic islets, which help in insulin metabolism (Borissova et al., 2003).

1.4.2.4b. Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is characterised by insulin resistance and progressive pancreatic β-cell dysfunction resulting in altered insulin secretion (Palomer et al., 2008; Song & Manson, 2010). Insulin resistance involves a loss of sensitivity of target cells to insulin. Hypovitaminosis D has long been suspected to be associated with metabolic syndrome and type 2 diabetes (Palomer et al., 2008). A London Bangladeshi population with very low levels of vitamin D have been reported at risk of type 2 diabetes compared to a British Caucasian population (Boucher et al., 1995). One of the factors that could prevent the onset of type 2 diabetes is sufficient levels of vitamin D in the body (Palomer et al., 2008). Vitamin D supplementation has improved insulin secretion and thus glucose tolerance in diabetic patients (Boucher et al., 1995). Thus, levels of vitamin D could play an important role in the early detection of diabetes (Palomer et al., 2008). In addition, another study showed that $25(OH)D_3$ levels were very low in newly diagnosed patients with type 2 diabetes or impaired glucose tolerance (Scragg et al. 1995). Often hypovitaminosis D is observed in patients with type 2 diabetes and obesity (Lee, Greenfield, Seibel, Eisman, & Center, 2009). Vitamin D is a fat soluble vitamin that is deposited in body fat where it is no longer bioavailable, which explains why many obese diabetes patients are vitamin D deficient (Palomer et al., 2008). This may then be accompanied by high levels of PTH, by the mechanism described earlier, giving rise to secondary hyperparathyroidism (Barone et al., 2007). Hyperparathyroidism contributes to impaired glucose tolerance and other cardiovascular diseases which in turn are also associated with obesity (Hagström et al.,

2009; Richards & Thompson, 1999). Insulin and PTH stimulate vitamin D production (Figure 1.3). Therefore, β cell dysfunction that results in acute insulin deficiency in diabetes may decrease vitamin D production and thus increase PTH levels giving rise to secondary hyperparathyroidism in these patients (Palomer et al., 2008).

1.4.2.4c. Immune response

Type 2 diabetes which may be the result of vitamin D deficiency is often accompanied by an increase in inflammatory factors such as tumour necrosis factor (TNF)-α and TNF-β, interleukin 6 (IL-6). These abnormalities observed in systemic inflammation markers may directly influence insulin signalling by several mechanisms, thus causing insulin resistance (Kolb & Mandrup-Poulsen, 2005). One of the mechanisms is the binding of these mediators to their receptors in muscle and hepatic tissues thereby interfering with phosphorylation of intracellular targets of the insulin receptor, dampening the response to insulin, and increasing insulin resistance (Senn et al., 2003). Thus, vitamin D deficiency which may lead to type 2 diabetes indirectly affects the immune system of the body and may give rise to insulin resistance. Apart from this role of vitamin D in the immune system, vitamin D promotes the differentiation of monocytes to macrophages and enhances phagocytosis by lysosomal enzymes (Hewison, Gacad, Lemire, & Adams, 2001). This function of vitamin D as an immune modulator is supported by the fact that VDR exists in activated T lymphocytes, macrophages and thymus tissue (Veldman, Cantorna, & DeLuca, 2000).

1.5. Mechanism of vitamin D action

Vitamin D as $1,25(OH)_2D$ acts in a homologous manner to other steroid hormones and exerts its biological activities within target cells *via* VDR which is a nuclear transcription factor (Bikle, 2009a; Walters, 1992). Thus, VDR is a ligand-induced transcription factor and binds through a carboxy-terminal ligand-binding domain to vitamin D (McDonnell, Scott, Kerner, O'Malley, & Pike, 1989). VDR belongs to the superfamily of nuclear receptors that include steroid, thyroid and retinoic acid receptors (Baker et al., 1988).

When $1,25(OH)_2D$ diffuses into the target cell, it binds to VDR and enhances the formation of heterodimers of VDR, generally with retinoid acid X receptor (RXR) (Almasan, Mangelsdorf, Ong, Wahl, & Evans, 1994) (Figure 1.4). The dimers activate or repress transcription of target genes by binding to either stimulatory (coactivators) or inhibitory (cosuppressors) coregulators whose varying amounts in different tissues could be responsible to an extent of responsiveness to $1,25(OH)_2D$ action and its production. Coactivator complexes generally either acetylate histones, thus exposing the DNA to be transcribed, or form a bridge between the initiation complex and dimer (as shown in Figure 1.4), enhancing transcription by RNA polymerase II. Co-repressors block this process of activation partly by deacetylating histones, although other mechanisms are also known (McKenna, Lanz, & O'Malley, 1999). The VDR-RXR dimers then bind to specific sequences in the target's promoter region which are called vitamin D response elements (*VDRE*). *VDRE* are present in genes involved in the regulation of calcium (Ca^{2+}) and phosphorus homeostasis and vitamin D metabolism and can be some distance away from the transcription initiation site (Kerner, Scott, & Pike, 1989). Some of these genes encode for Ca^{2+} transporting proteins and bone-forming enzymes, such as calbindin D_{9k}, osteocalcin and osteopontin (Kerner et al., 1989). $1,25(OH)_2D$ also controls cell proliferation and differentiation by up-regulating inhibitors of cyclin dependent kinases, p21 and p27. Low levels of these inhibitors are common in many tumour types (Carlberg & Seuter, 2009). It also reduces the risk of cancer by preventing angiogenesis, thereby reducing nutrition to a malignant cell (Holick, 2008). $1,25(OH)_2D$ down-regulates a comparable number of genes as it up-regulates. The mechanism of down-regulation is poorly understood but it has been postulated that these genes require the binding of a VDR agonist (Carlberg & Seuter, 2009). The down-regulation of 1α-hydroxylase (CYP27B1) by $1,25(OH)_2D$ involves a negative *VDRE* located at position -0.5 kB, where VDR-RXR heterodimers do not bind directly, but bind *via* a transcription factor VDR-interacting repressor. This way $1,25(OH)_2D$ controls its own production by down regulating *CYP27B1* gene (Turunen, Dunlop, Carlberg, & Vaisanen, 2007).

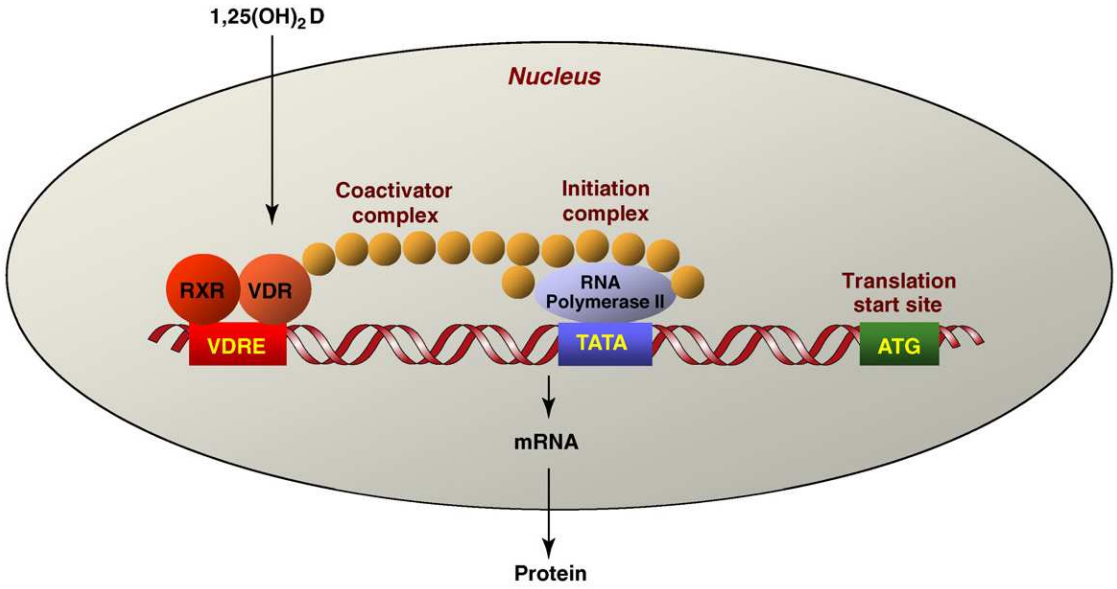


Figure 1.4. The mechanism of action of $1,25(OH)_2D$ mediated by VDR: When $1,25(OH)_2D$ enters the cell, it binds to VDR, transporting it to the nucleus and facilitating dimer formation of VDR primarily with RXR. These dimers bind to specific regions of the target genes called *VDRE*, accompanied by the formation of large complexes that can activate (coactivators) or inhibit (cosuppressors) gene transcription.

From "Vitamin D: newly discovered actions require reconsideration of physiologic requirements," by Daniel D. Bikle 2010, *Trends in Endocrinology and Metabolism*, p.4. Copyright© 2010 by Elsevier Ltd.

1.6. Vitamin D receptor (*VDR*) gene

The *VDR* gene is large at just over 100 kbp and is located on chromosome 12q13, downstream from the collagen type II alpha 1 (*COL2A1*) gene (Uitterlinden, Fang, Meurs, Pols, & Leeuwen, 2004) (Figure 1.5). The region upstream of exon 1a of the *VDR* gene contains GC-rich islands but does not contain an obvious TATA box (Miyamoto et al., 1997). The *VDR* gene consists of fourteen exons (1-9), of which 1a-1f are in the regulatory region capable of generating multiple tissue-specific transcripts through alternative splicing and/or alternative promoter usage and the remaining eight exons are protein-coding exons (2-9) (Crofts, Hancock, Morrison, & Eisman, 1998) (Figure 1.5). Due to alternative splicing and/or alternative promoter usage, VDR transcript levels may vary from tissue to tissue as a subset of the mRNA may be expressed in one tissue but not the other (Crofts et al., 1998). This could be the reason for the high levels of *VDR* gene expression in metabolic tissues, such as intestine and kidney, as well as in skin and the thyroid gland, but moderate

levels of expression in nearly all the tissues (Carlberg & Seuter, 2009). The two zinc finger DNA binding motifs that help VDR to bind to *VDRE* in the target genes are encoded by exons 2 to 4. The ligand binding domain that interacts with $1,25(OH)_2D$ is encoded by exons 6 to 9 (Rukin & Strange, 2007).

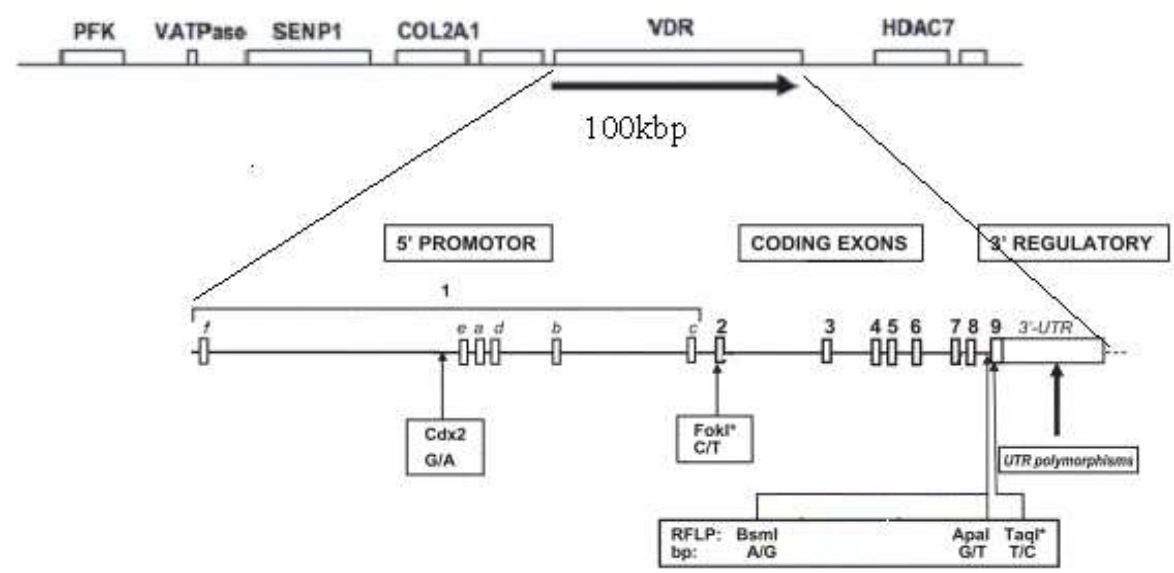


Figure 1.5. Location of the *VDR* gene on chromosome 12, its exon-intron structure and position of the five polymorphisms analysed in this study. * indicates that the polymorphism is in the coding sequence.

Modified from "Genetics and biology of vitamin D receptor polymorphisms," by Andre G. Uitterlinden, Yue Fang, Joyce B.J. van Meurs, Huibert A.P. Pols and Johannes P.T.M. van Leeuwen 2004, *Gene*, *338*, p.145,146. Copyright © 2004 by the Elsevier Science B.V.

1.7. Single nucleotide polymorphisms (SNPs)

Variations in the DNA sequence of the coding or non-coding regions by a single base (single nucleotide polymorphisms, SNPs) or a variable number of repeats of a short repetitive DNA sequence (variable number of tandem repeats, VNTRs), have the potential to regulate transcripts or protein levels and/or change the sequence of amino acids in the protein (Ogunkolade et al., 2002). As a consequence, SNPs have been responsible for, and have been linked to several diseases such as osteoarthritis (OA), diabetes, cancer etc (Bid, Mishra, & Mittal, 2005; Uitterlinden et al., 2004). A classic example of a SNP influencing

the protein product is that of haemoglobin and the resulting sickle cell anaemia (Chang & Kan, 1981). Deleterious mutations in the coding region of *VDR* cause a rare autosomal recessive form of vitamin D-resistant rickets in the homozygous individuals (Reis, Hauache & Velho, 2005). More than nine million SNPs have been recorded in the human genome and stored in public databases (Kim & Misra, 2007). These subtle variations in DNA have been reported as important markers that link them to phenotypic changes or they may be in close proximity or linkage disequilibrium to a mutant gene that causes the disease (Kwok, 2001). Linkage disequilibrium (LD) describes the 'association of alleles of adjacent polymorphisms with each other' (Uitterlinden et al., 2004, p.147). The combination of SNPs on a DNA fragment is known as a haplotype, which has been used in genome-wide association studies. For this reason, there is a current focus on advancing and developing SNP genotyping techniques to accurately discriminate between alleles (Kim & Misra, 2007).

Allelic discrimination strategies are described in sections 2.1.5 and 3.1.1.

1.7.1. SNPs in the *VDR* gene

Recent studies have suggested that SNPs may influence the stability, quantity and activity of the VDR protein and rate of VDR mRNA formation (Ogunkolade et al., 2002). The possession of specific alleles may in turn have an impact on the severity and susceptibility to the several disorders mentioned above. Interest in *VDR* SNPs has increased in recent years and substantial progress in understanding the association of the *VDR* SNPs and risk assessment of disease has been made (Uitterlinden et al., 2004). To date, more than 470 SNPs have been reported in the *VDR* gene and their combination that an individual inherits gives them a specific haplotype which may determine the predisposition to hypovitaminosis D and its downstream effects (Rukin & Strange, 2007). The relative positions of five SNPs (Cdx-2, *Fok*I, *Bsm*I, *Apa*I and *Taq*I) are shown in Figure 1.5. Cdx-2 is present upstream of exon 1e, *Fok*I is present in exon 2, *Bsm*I and *Apa*I polymorphisms in intron 8 (between exons 8 and 9) and *Taq*I in exon 9 (Quesada et al., 2004). These five polymorphisms have been extensively studied to find their associations with various diseases such as insulin

resistance, autoimmune disorders etc (Borissova et al., 2003; Chiu, Chuang, & Yoon, 2001; Eccleshall, Garnero, Gross, Delmas, & Feldman, 1998). The SNP identification number for each SNP is shown in Table 1.1.

Table 1.1. Individual SNPs and their SNP identification numbers

Individual SNP	*SNP identification number*
Cdx-2	rs11568820
*Fok*I	rs10735810
*Bsm*I	rs1544410
*Apa*I	rs7975232
*Taq*I	rs731236

From "Single nucleotide polymorphism". Retrieved November 15, 2009, from http://www.ncbi.nlm.nih.gov/projects/SNP/

Most allelic variants that have been studied so far have been analysed by amplifying the gene by the polymerase chain reaction (PCR) and digesting with restriction enzymes for polymorphic banding patterns, a technique known as restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) (Uitterlinden et al., 2004). This technique was used to discover *VDR* gene polymorphisms such as those found at the *Fok*I (Gross et al., 1996), *Bsm*I, *Apa*I and *Taq*I restriction enzyme sites (Morrison et al., 1994). However, Cdx-2 SNP was found through sequence analysis of a targeted area (Arai et al., 2001). The absence of a restriction enzyme site is denoted by an uppercase letter (F, B, A, T) and the presence of the site is denoted by a lowercase letter (f, b, a, t). Thus, homozygous individuals who do not have the restriction enzyme sites are denoted by FF (*Fok*I), BB (*Bsm*I), AA (*Apa*I), TT (*Taq*I) genotypes whereas individuals who have the restriction sites are denoted by ff, bb, aa and tt genotypes.

These five polymorphisms are described further in chapter 2.

1.8. Vitamin D-binding protein (DBP)

Vitamin D binding protein (DBP) is the major plasma carrier of vitamin D protein. It is a 52-59 kDa protein that has important biological functions apart from acting as a carrier for vitamin D. These functions include fatty acid transport, macrophage activation and chemotaxis and actin scavenging action (Speeckaert, Huang, Delanghe, & Taes, 2006). It is encoded by the gene *Gc* which is located at chromosome 4q11-q13 and belongs to the family of albumin and alpha-fetoprotein genes (McCullough, Bostick & Mayo, 2009). Studies on knock-out mice demonstrated the importance of this protein in carrying vitamin D metabolites to various tissues and sites of action (Reis et al., 2005). The *Gc* gene is 42.5 kb long and has 13 exons. There are at least six nonsynonymous SNPs of which two are the most frequent (*rs7041* and *rs4588*) (McCullough et al., 2009). The polymorphic variants of this protein affect the protein's binding affinity to $1,25(OH)_2D$ (Arnaud & Constans, 1993). Several studies have investigated associations of polymorphisms of this protein to type 2 diabetes, insulin levels or glucose tolerance (Hirai et al., 2000; Malecki et al., 2002). Another study had shown an association of the C/T polymorphism at position -39 in the *Gc* promoter region with bone mineral density (BMD) in Japanese adult women (Ezura et al., 2003).

1.9. The Surya Study and the present study

A previous study, the 'Surya Study', by Hurst et al. (2008) focussed on establishing the vitamin D status in 239 South-Asian women who were ≥20 years old and were living in Auckland (Phase 1). These women had serum 25(OH)D levels less than 80 nmol/L. They were then screened for entry into the intervention phase that required serum 25(OH)D < 50 nmol/L and high insulin resistance (HOMA-IR>1.93). Eighty–one women entered the intervention based on the above criteria and completed a six-month randomised controlled trial where forty-two of them were given 4000 IU vitamin D_3 supplements daily and thirty-nine were given a placebo (Phase 2). Their responsiveness to the intervention was assessed by endpoint measurements such as insulin resistance/ sensitivity, fasting C-peptide etc.

The research reported here is an extension of the Surya Study and looked at the five polymorphic variants in the *VDR* gene carried by each participant and the potential association of these variants with insulin resistance (HOMA-IR) and insulin sensitivity (HOMA2%S). The genotypes for each of the five SNPs (Cdx-2, *Fok*I, *Bsm*I, *Apa*I and *Taq*I) were determined by allele-specific multiplex PCR (Cdx-2) and RFLP-PCR (*Fok*I, *Bsm*I, *Apa*I and *Taq*I). Haplotypes were determined for *Bsm*I, *Apa*I and *Taq*I using RFLP-PCR. The biological aim of this research was complemented by a technical aim in comparing real-time PCR and RFLP-PCR in identifying three SNPs at the 3' end of the *VDR* gene (*Bsm*I, *Apa*I and *Taq*I). The frequency of each SNP and haplotype was determined for this cohort of South-Asian women living in Auckland.

An outline of this study is shown Figure 1.6.

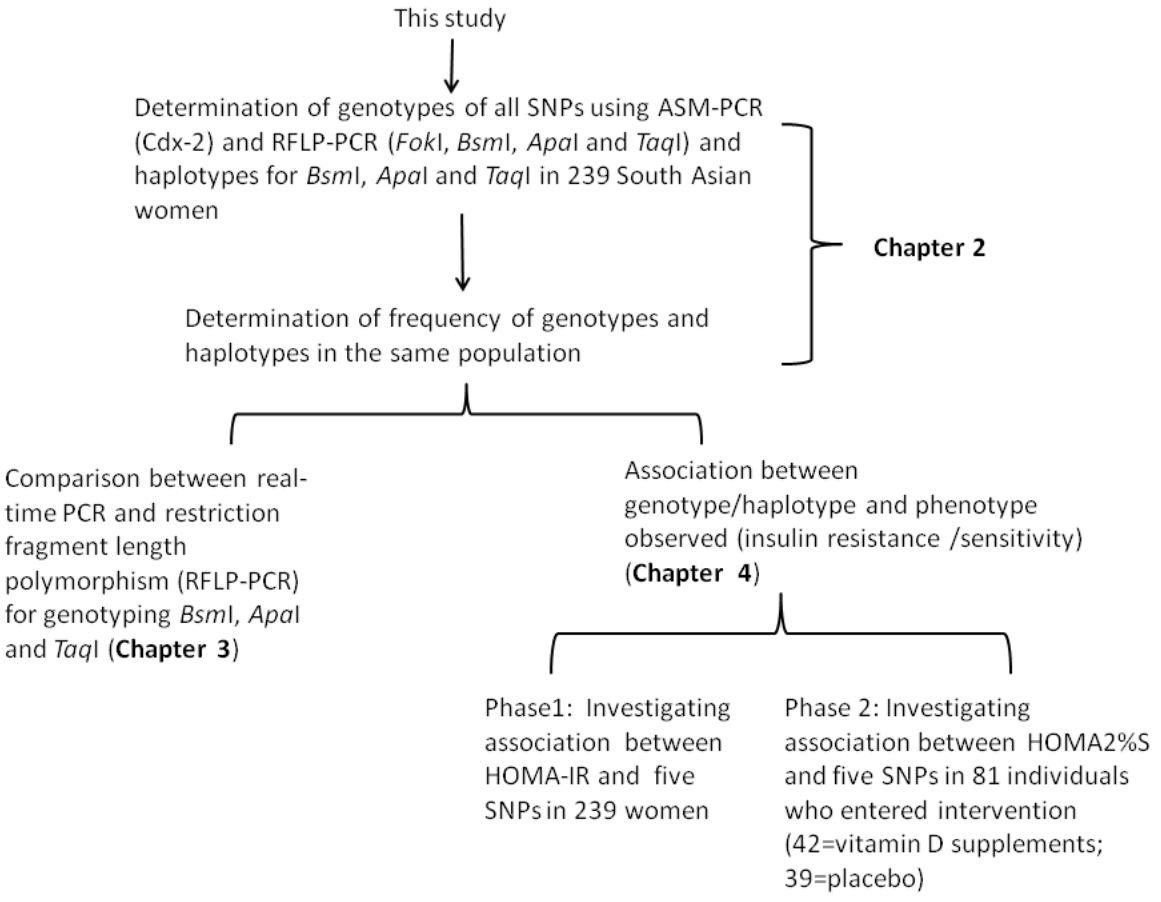


Figure 1.6. A schematic representation of the outline of the present study.

Chapter 2

Methods & Materials, Results & Discussion: Genotype and Haplotype analysis of the *VDR* SNPs

2.1. Introduction

The potential role of SNPs in human diseases has already been described in chapter 1. Most of the research on *VDR* gene SNPs has focussed on the five SNPs (Cdx-2, *Fok*I, *Bsm*I, *Apa*I and *Taq*I) which have been extensively studied for their associations to diseases such as osteoporosis, diabetes, cancer etc. Each SNP is described in turn below.

2.1.1. Cdx-2 SNP

The Cdx-2 SNP was identified by sequence analysis of the promoter region. Arai et al. (2001) reported a G to A transition located 3731 bp upstream of the exon 1a promoter region of the *VDR* gene aomng Japanese women. However, Fang et al. (2003) showed that the 1e promoter region was just 2 kbp away from 1a and that this transition in the 1a promoter region was in fact present 1739 kbp upstream of 1e region in many ethnic populations (1e-G-1739A) (Figure 1.5). The Cdx-2 SNP is in the binding site region of the transcription factor Cdx-2, a caudal-related homeodomain protein that increases intestinal-specific transcription of the *VDR* gene leading to an increase in calcium absorption from the intestine (Jehan, d'Alesio, & Garabedian, 2007). When the A allele is present at the Cdx-2 locus, Cdx-2 transcription factor binds more strongly than when a G nucleotide is present (G allele). This stronger binding increases transcription of the *VDR* gene which in turn increases calcium absorption in the intestine (Arai et al., 2001). The G allele disrupts the Cdx-2 transcription-factor binding site which in turn decreases *VDR* gene transcription (Arai et al., 2001). Therefore, the A allele increases production of calcium transport proteins by increasing transcription of the *VDR* gene which in turn affect calcium uptake. This outcome influences BMD and susceptibility to fractures in the elderly (Fang et al., 2003; Jehan et al., 2007; Lins et al., 2007; Uitterlinden et al., 2004). Individuals homozygous for A and G alleles have AA and GG genotypes and heterozygous individuals have AG genotype.

2.1.2. *Fok*I SNP

*Fok*I polymorphism is also referred to as the start codon polymorphism (SCP) and is a T to C polymorphism that alters the start codon resulting in a more active VDR protein that is three amino acids shorter (Lins et al., 2007; Rukin & Strange, 2007). The absence of the *Fok*I restriction enzyme site (denoted by F) results in the translation of this shorter 424-amino acid residue protein (also referred to as the M4 form) that is initiated at the Met-4 position whereas the presence of the *Fok*I site (denoted by f) results in translation initiation at the Met-1 position and a VDR protein of 427 amino acids is formed (also referred to as the M1 form) (Rukin & Strange, 2007; Uitterlinden et al., 2004). The restriction recognition site of *Fok*I is 5'-GGATG*-3'; 3'-CCTAC*-5' in duplex DNA and the enzyme cleaves 9/13 nucleotides downstream of the recognition site. Thus, homozygous individuals with the restriction enzyme site have TT genotype (ff); homozygous individuals without the restriction enzyme sites have CC genotype (FF) and heterozygous individuals have TC genotype (Ff).

2.1.3. *Bsm*I-*Apa*I-*Taq*I SNPs

Most efforts to identify functional sequence variants in the *VDR* gene have focussed on polymorphisms at the 3' end (*Bsm*I, *Apa*I and *Taq*I) which are in linkage disequilibrium (LD) with each other and may have the potential to influence the stability of the VDR mRNA (Davis, 2008; Fang et al., 2006). It was reported that *Apa*I and *Taq*I are tightly linked whereas *Bsm*I has relatively low linkage disequilibrium to these two (Fang et al., 2006). Therefore, even though *Bsm*I, *Apa*I and *Taq*I are located in the same haplotype block, association studies of *Apa*I and *Taq*I can be merged as one genotype group but cannot be merged with studies on *Bsm*I (Fang et al., 2005, Fang et al., 2006). The *Fok*I and Cdx-2 SNPs do not appear to be linked with the *Bsm*I-*Apa*I-*Taq*I polymorphisms (Fang et al., 2006). It is still unclear how the *Bsm*I and/or *Apa*I SNPs located in the non-coding regions of the *VDR* gene and the synonymous *Taq*I variants might influence *VDR* function and associated diseases. It is possible that these polymorphisms are in LD with as yet unidentified functionally important allelic sites located elsewhere in the *VDR* gene, or to a

nearby gene that plays a role in phenotypic traits or diseases and they can therefore be used as markers (Miyamoto et al., 1997; Uitterlinden et al., 2004). However, the linkage between the marker allele and the functional allele depends on the distance that influences their strength of LD that in turn can lead to varying associations (Uitterlinden et al., 2004).

The presence of a restriction enzyme site in these SNP sites is denoted by an uppercase letter and the absence by a lowercase letter. AA (absence of restriction site) for *Apa*I should not be confused with AA of Cdx-2. The recognition site for *Bsm*I is 5'-GAATGCN*-3', *Apa*I 5'-GGGCC*C-3' and *Taq*I 5'-T*CGA-3'. Thus, homozygous individuals with restriction enzyme sites for *Bsm*I, *Apa*I and *Taq*I have GG, CC and CC genotypes, respectively, and those who do not have the site for the above SNPs have the corresponding AA, AA and TT; heterozygous individuals have genotypes GA, GT and CT, respectively. Therefore, an individual with a haplotype BAT has no restriction sites for *Bsm*I, *Apa*I and *Taq*I. Other haplotypes are described later in this chapter.

A summary of these five SNPs, their restriction sites, SNP sequences and zygosity is described in the Table 2.1.

Table 2.1. A summary of the SNPs studied for this project with regards to their restriction sites, SNP sequences and their zygosity

SNP	*SNP sequence (5'-3')^*	*Restriction site*	*Zygosity*					
			Homozygous 1		*Heterozygous*		*Homozygous 2*	
			Genotype	*Nucleotides at the SNP site on both the chromosomes*	*Genotype*	*Nucleotides at the SNP site on both the chromosomes*	*Genotype*	*Nucleotides at the SNP site on both the chromosomes*
Cdx-2	GGTCACA[A/G]TAAAAAC	N.A	AA	N.A	AG	N.A	GG	N.A
*Fok*I	TGCCTCC[T/C]TCCCTGT	5'-GGATG*-3' 3'-CCTAC*-5'. Cleaves 9/13 nucleotides downstream of the recognition site	FF†	CC	Ff†	TC	ff†	TT
*Bsm*I	GCCTGC[A/G]CATTCC	5'-GAATGCN*-3'	BB†	AA	Bb†	AG	bb†	GG
*Apa*I	GCTGGGC[A/C]CCTCACT	5'-GGGCC*C-3'	AA†	AA	Aa†	AC	aa†	CC
*Taq*I	CGCTGAT[C/T]GAGGCCA	5'-T*CGA-3'	TT†	TT	Tt†	TC	tt†	CC

†Uppercase letter indicates absence of the restriction enzyme site, lowercase presence of a restriction enzyme site
^ From "Human Genome Variation Genotype-to-Phenotype database (HGVbaseG2P)". (2009). Retrieved May 21, 2010, from http://www.hgvbaseg2p.org/index
N.A.-not applicable

2.1.4. Frequency of SNPs in different populations

Some of the aforementioned polymorphisms in the *VDR* gene are present at very low frequencies within different populations, making the study of these individual SNPs impractical, difficult and time consuming (Rukin & Strange, 2007). For example, Figure 2.1 shows that only 7% of Asians had a SNP at a *Bsm*I restriction enzyme site whereas 74% of Asians had a SNP within an *Apa*I restriction enzyme site. In general, polymorphisms start as random mutations that may eventually grow in proportion in a particular ethnic group to become true polymorphisms. These evolutionary processes and population genetic behaviour are likely to explain for allele frequency differences between different ethnic groups (Uitterlinden et al., 2004).

There appears to be marked variations in the type and frequency of VDR gene haplotypes that exist in different ethnic groups. Also, these differences may be responsible for the variation in disease status seen between different ethnic groups (Uitterlinden et al., 2004). For example, Figure 2.1 shows the frequencies of the *Bsm*I-*Apa*I-*Taq*I *VDR* SNP haplotypes in different ethnic groups. They show a complex pattern of frequencies which cannot be derived by calculating frequencies of individual polymorphisms. The reason for this discrepancy may be that when many SNPs exist in a gene, the information related at one site is 'linked' to its neighbour in a block (haplotype block) and this means that relatively few SNPs have to be genotyped to study large portions of a gene (Thakkinstian, D'Este, Eisman, Nguyen, & Attia, 2004).

VDR gene polymorphism [a]	Minor alelle	Ethnic Group (%)		
		Caucasian	Asian	African
Individual polymorphism				
Cdx-2	A	19	43	74
*Fok*I	f ~ T	34	51	24
*Bsm*I	B	42	7	36
*Apa*I	A	44	74	31
*Taq*I	T	43	8	31
Bsm-Apa-Taq haplotypes				
	baT: haplotype 1	43	75	26
	BAt: haplotype 2	39	7	16
	bAT: haplotype 3	11	17	59

[a] Data are from various sources (Arai et al., 1997; Durin et al., 1999; Fan et al., 2003; Franco et al., 1989; Gross et al., 1996; Ingles et al., 1997; Sturzenbecker et al., 1994; Uitterlinden et al., 1996; Ye et al., 2000) and from Fan et al. (manuscript in preparation)

Figure 2.1. Comparison of the frequencies of the most widely studied polymorphisms across the three major ethnic groups. Uppercase B, A or T refers to absence of the restriction enzyme site *Bsm*I, *Apa*I and *Taq*I respectively. Lowercase refers to the presence of the site. However, no restriction enzyme site is present at the Cdx-2 site and so A represents the actual nucleotide at the SNP site.

2.1.5. Allelic discrimination strategies

To accurately identify the alleles, an ideal genotyping method must possess the following attributes: (a) the assay should be quick and reliable; (b) it should be cheap; (c) it should involve limited optimisation and analysis time (Kwok, 2001). Allelic discrimination strategies rely on four popular methods: primer extension, enzymatic cleavage, ligation and

hybridisation of which three strategies are described below and the rest is described in section 3.1.1.

2.1.5.1. Primer extension

Primer extension is a very simple technique. It is usually straightforward with regard to primer design, and a large number of SNPs can be analysed simultaneously (Kwok, 2001; Kim & Misra, 2007). This technique relies on allele-specific incorporation of nucleotides by DNA polymerase on template DNA. Primer extension techniques can be grouped into two categories: one uses common primers and other uses allele-specific primers to achieve allelic discrimination (Kim & Misra, 2007) (Figure 2.2).

In a common primer extension (CPE), the designed primer anneals one base upstream of the SNP site and relies on incorporation of the nucleotide by DNA polymerase at the SNP site. This nucleotide at the SNP site is then detected by either mass spectroscopy or by fluorescence (Kim & Misra, 2007) (Figure 2.2a and b). In mass spectroscopy, after the primer anneals one base upstream of the SNP site, it is extended with ddNTPs inhibiting further extension. The nucleotide at the SNP site is then detected by the difference between the mass of the primer and the extended product (Ross, Hall, Smirnov, & Haff, 1998) (Figure 2.2a). In the case of fluorescence detection, when the primer anneals one base upstream of the SNP site, it is extended at the SNP site by ddNTPs that are fluorescently labelled with different dyes (Figure 2.2b). The fluorescence of the product is then captured and the colour of the amplicon indicates the incorporated nucleotide at the SNP site (Hellard et al., 2002; Sokolov, 1989).

The specific primer extension (SPE) approach uses two allele-specific primers that differ only at their 3' end. DNA polymerase extends the primer bound to the template DNA only when it is perfectly complementary to the DNA at its 3' end (Kim & Misra, 2007; Kwok, 2001). One can infer which allele is present by determining whether a product is formed or not by gel electrophoresis (Kwok, 2001). Allele-specific PCR (AS-PCR) uses allele-specific primers that have different labels and a common reverse primer to achieve allelic

discrimination and the product can be analysed by fluorescence (Gibbs, Nguyen, & Caskey, 1989) (Figure 2.2c).

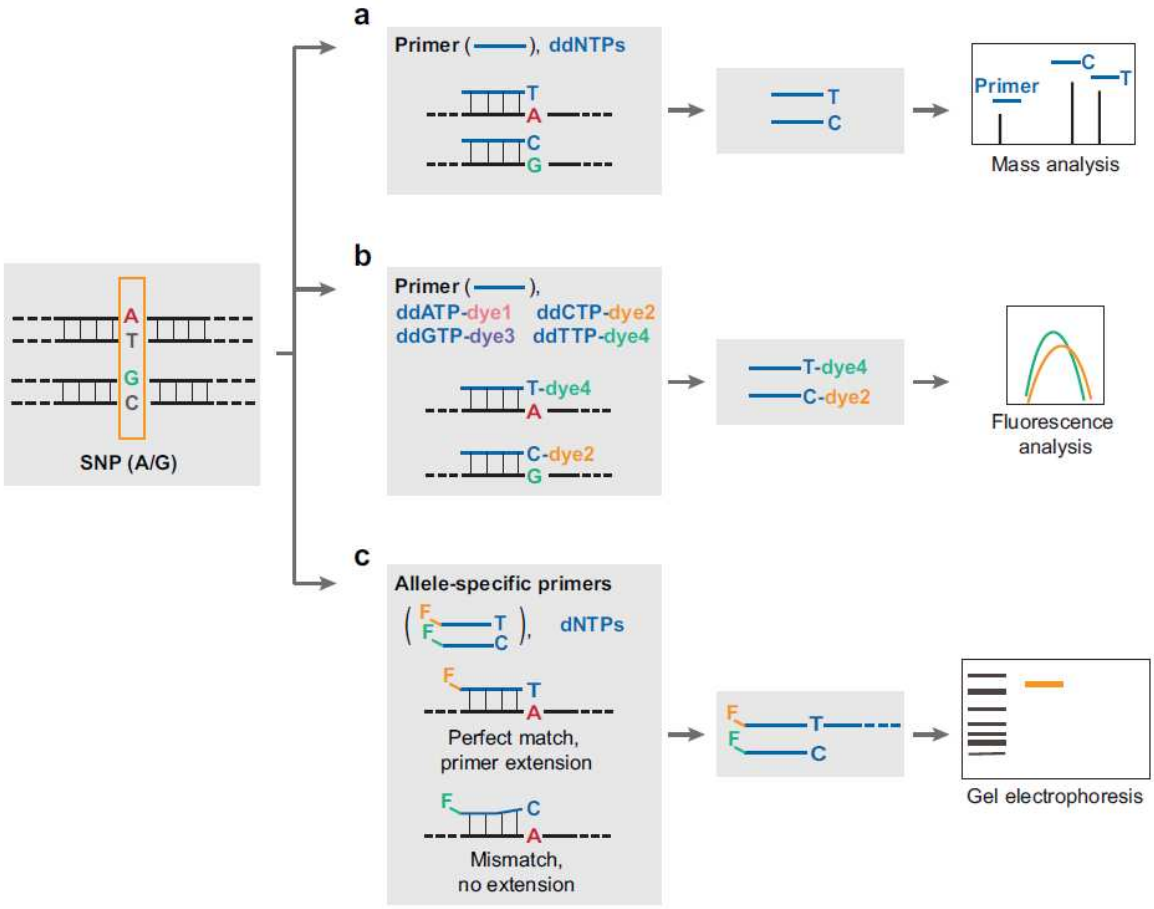


Figure 2.2. Primer extension technique: Common primer extension (CPE)-uses a common primer for the two alleles which bind to the complementary sequence on DNA adjacent to the SNP site. The discrimination is then achieved by the incorporation of a nucleotide by DNA polymerase at the SNP site, which is detected by (a) mass spectroscopy or (b) fluorescence. (c) Specific primer extension (SPE)- uses two primers that are specific to alleles at their 3' end. Only when primer binds at the SNP site with perfect complementarity that a PCR product is formed which can be detected by gel electrophoresis.

From "SNP Genotyping: Technologies and Biomedical Applications," by Sobin Kim and Ashish Misra 2007, *Annual Review of Biomedical Engineering, 9*, p.291. Copyright ©2007 by Annual Reviews.

2.1.5.2. Enzymatic cleavage

This approach relies on the ability of certain classes of enzymes to recognise and cleave specific sequences and structures. Such enzymes can be used to cleave at SNP sites thus discriminating between alleles (Kim & Misra, 2007).

A traditional method for discrimination of alleles is restriction fragment length polymorphism PCR (RFLP-PCR). Restriction enzymes recognize specific sequences on target DNA and cleave both strands at the sequence or near to it. In genotyping SNPs, these enzymes are incubated with the DNA or PCR product containing the SNP at a particular temperature (Botstein, White, Skolnick, & Davis, 1980). If the restriction site corresponds to a specific allele which is present, the enzyme cleaves at the SNP location. Allelic discrimination is achieved by the presence or absence of restriction product/s that is visualized by gel electrophoresis. SNP determination is easily done by analysing the product sizes (Figure 2.3). This method does not require probes; however, it is a time-consuming procedure that has limited throughput and is applicable only to SNPs that occur at a restriction enzyme site (Kim & Misra, 2007).

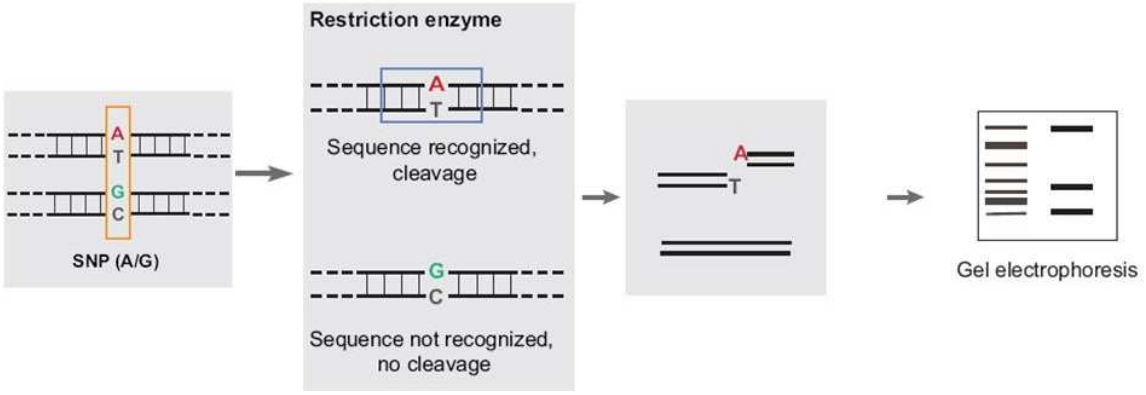


Figure 2.3. Cleavage by restriction enzyme. This approach uses a restriction enzyme that cleaves one of the alleles. The digested products are separated by gel electrophoresis and the sizes of products are determined.

Modified from "SNP Genotyping: Technologies and Biomedical Applications," by Sobin Kim and Ashish Misra 2007, *Annual Review of Biomedical Engineering*, *9*, p.291. Copyright ©2007 by Annual Reviews.

2.1.5.3. Ligation

Ligation assays rely on the ability of DNA ligases to repair nicks in DNA. Ligase requires that the two nucleotides it joins (by creating a phosphodiester linkage) are base paired to their complementary sequences. This approach uses two allele-specific probes and another common probe that anneals adjacent to the SNP site, next to the allele specific probe (Kim

& Misra, 2007) (Figure 2.4). Only when the allele-specific probe binds to the SNP site with perfect complementarity, the ligase enzyme joins it with the common probe which has annealed just adjacent to the SNP site (Landegren, Kaiser, Sanders, & Hood, 1988). These two allele-specific probes can have two different fluorescent dyes and the formation of product is determined by fluorescence of the product. This determines the presence of the allele (Tong, Li, Jones, Russo, & Ju, 2001). Mostly, the allele-specific probes have the mismatched base at the 3' end that gives them allele specificity (Kim & Misra, 2007) (Figure 3.4). Although ligation is highly specific and requires less time to optimize as compared to other allelic discrimination techniques, it is the slowest reaction (Kwok, 2001).

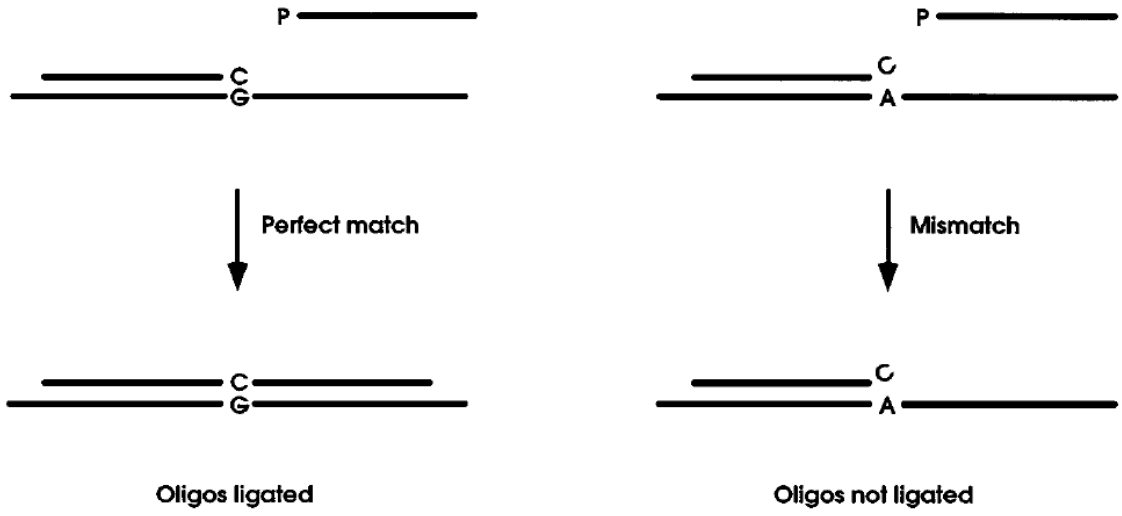


Figure 2.4. Allele-specific ligation: relies on ligation of two probes, one of which needs to perfectly match the SNP site and the other anneals at an adjacent site.

From "Methods and genotyping single nucleotide polymorphisms ," by Pui-Yan Kwok 2001, *Annual Review of Genomics and Human Genetics, 2*, p.238,239. Copyright ©2001 by Annual Reviews

2.1.6. Haplotyping methods

Haplotype is the combination of alleles of different SNPs on a chromosome. Since humans are diploid, each individual has two haplotypes, representing maternal and paternal chromosomes (Crawford & Nickerson, 2005). Many studies have used various approaches to determine haplotype of an individual (Fang et al., 2005; Fang et al., 2007; Uitterlinden et al., 1996). These methods to determine haplotype can be broadly divided into two

categories: Statistical methods and experimental methods. An approach used by the statistical methods is the prediction of SNP pattern in certain genetic/haplotype blocks. This means that for some blocks, only few combinations of alleles/haplotypes exist as the alleles are linked to each other in a haplotype block, that is, these combinations will then account for 80-90% of variation in the entire population (Smith, 2002).

Several molecular or experimental methods are available for identifying SNPs and constructing haplotypes. These techniques include AS-PCR, RFLP-PCR and somatic cell hybrids (Crawford & Nickerson, 2005). The AS-PCR technique as described earlier can be used to detect genotypes and possibly haplotypes by designing primers that amplify one of the two alleles of the desired marker. The resulting product is then subjected to genotyping of the second marker. Thus, AS-PCR requires several rounds of PCR, is expensive and time consuming (Michalatos-Beloin, Tishkoff, Bentley, Kidd, & Ruano, 1996). RFLP-PCR can also be used for determining haplotype; a PCR product can be generated that spans more than one SNP and the product is digested with restriction enzymes that recognize the SNP sites. As a result, a complex fragment pattern may be used to determine haplotype on each chromosome (Uitterlinden et al., 1996). However, both AS-PCR and RFLP-PCR are limited by the distance between the SNPs that can be amplified and the requirement of a SNP at a restriction site is another disadvantage of RFLP-PCR (Michalatos-Beloin et al., 1996; Uitterlinden et al., 1996). The somatic cell hybrid technique physically separates maternal and paternal chromosomes of an individual before haplotyping. However, it is an expensive and laborious technique (Patil et al., 2001). Nevertheless, analysis of haplotype is important in determining the functional relevance of an allele (Ragoussis, 2007). It also yields information about the recombination events occurring during meiosis and which alleles are linked and which are not. Information about recombination is important for locating disease-causing mutations that could be linked to a nearby gene (Crawford & Nickerson, 2005). Thus, if the genotype at a SNP site is known, it could be used to predict the disease-causing mutation (Crawford & Nickerson, 2005).

This chapter outlines all common procedures for genotyping and haplotyping. Procedures specific to different analyses are outlined in the relevant chapters.

2.2. Methods and Materials

2.2.1. gDNA extraction for RFLP-PCR and ASM-PCR

Since the research described here is an extension of the Surya Study, buffy coats were already collected from the participants. Genomic DNA (gDNA) was extracted from buffy coat samples using the Invitrogen Purelink Genomic DNA kit. For each sample, 20 µl, 50 µl and 100 µl of buffy coat were adjusted with 180 µl, 150 µl and 100 µl of phosphate buffer saline (PBS), respectively, and gDNA was extracted according to the manufacturer's instructions with the following alterations to increase the yield. Firstly, the incubation time for protein digestion at 55^{o}C was increased from 10 min to 15 min. Secondly, the incubation time for the elution step was increased from 1 min to 2 min. The purified DNA was eluted in 50 µl of elution buffer and stored at -20 ^{o}C. Each volume of buffy coat gave good quality gDNA. However, 50 µl of buffy coat was considered optimal for the yield and number of assays to be performed, thus this volume was used routinely.

2.2.2. RFLP-PCR genotyping/haplotyping of *Bsm*I, *Apa*I and *Taq*I SNPs

The RFLP-PCR method of Uitterlinden et al. (1996) was adapted for the analysis of the *Bsm*I, *Apa*I and *Taq*I SNPs in 239 individuals. The primers VDR1 (5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3') and VDR4 (5'-GCAACTCCTCATGGCTGAGGTCTC-3') were used to amplify a 2229 bp PCR product which included all three SNPs. PCR was carried out in a 75 µl reaction containing 6 µl of gDNA, 35.5 µl of 2X GoTaq® Green Master Mix (Promega) and 3 µl each of 10 µM VDR1 and VDR4. The thermal cycling conditions were carried out in a Techne TC-512 machine as follows: 95^{o}C for 2 min, 94, 60 and 72^{o}C for 1 min each, for 30 cycles and a final extension of 72^{o}C for 10 min. The PCR products were analysed in a 1% agarose gel in a 1X TAE buffer system at 75V for 1.5 hours. This product was digested with each restriction enzyme separately to reveal the genotype for each SNP and in combination to give the haplotype of the individual.

The PCR product (10 µl) was digested individually in three separate tubes with 1.25U each of *Bsm*I (New England Biolabs), or *Taq*I (Roche) or 10U of *Apa*I (Roche) with 1.25 µl of 10X restriction buffer (150mM Tris-HCl, pH 7.5, 250mM NaCl and 35mM $MgCl_2$) and incubated at 31°C for 45 min for *Apa*I and at 65°C for 45 min for *Bsm*I and *Taq*I (Uitterlinden et al., 1996). The PCR product (20 µl) was also digested with 2.5U of each of *Bsm*I, *Apa*I and *Taq*I, and 2.5 µl of the 10X restriction buffer. The products were incubated for 45 min at 31°C for digestion with *Apa*I and then for 45 min at 65°C for digestion with both *Bsm*I and *Taq*I. Digestion products were analysed in a 1.4% agarose gel run in 1X TAE for 2.5 hours at 70V.

The alleles were named according to the convention in the literature; the absence of a restriction enzyme site was denoted by an uppercase letter and the presence of restriction enzyme site was denoted by a lowercase letter.

The PCR product of few samples were sent for sequencing to Ecogene™ DNA based Diagnostics for *Bsm*I, *Apa*I and *Taq*I genotypes. The genotypes were confirmed by sequencing on a ABI 3100-Avant Genetic Analyzer (Applied Biosystems) using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequence analysis was done using software package named Geneious Pro v.4.7 (Drummond, 2009). The peaks at SNP positions were analysed for evidence of zygosity for these samples.

2.2.3. RFLP-PCR genotyping of *Fok*I SNP

The *Fok*I SNP was analysed by RFLP-PCR using a modification of the method described by Rezende et al. (2007). The primer sequences were

*Fok*I Fwd: 5'-GATGCCAGCTGGCCCTGGCACTG-3'
*Fok*I Rev: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'
PCR was carried out using gDNA isolated from buffy coat taken from each participant using the Invitrogen Purelink Genomic DNA kit as described in section 2.2.1. The PCR was performed in a 15 µl reaction volume containing 0.3 µl of each 10 µM forward and reverse primers, 3 µl of template gDNA and 7.5 µl of GoTaq® Green Master Mix

(Promega). The PCR was carried out in a Techne TC-512 machine and the thermal conditions were: initial denaturation at 95^{o}C for 5 min followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 63^{o}C for 30 sec and extension at 72^{o}C for 30 sec. Final extension was conducted at 72^{o}C for 3 min. The expected length of the PCR product was 272 bp.

PCR product (10 µl) was digested with 10U of *FokI* (New England Biolabs) enzyme at 37^{o}C overnight with 2 µl of restriction buffer supplied with the enzyme in a total volume of 20 µl. The PCR and digested products were separated in a 2.5% agarose gel using 0.5X TBE buffer system at 120V for 45 min.

2.2.4. ASM-PCR genotyping of Cdx-2 SNP

For the amplification of the Cdx-2 SNP, the two primers specific to the SNP were A-For and G-Rev while the two primers that flanked the SNP site were A-Rev and G-For (Fang et al., 2003). Amplification with A-Rev and G-For generated a 297 bp fragment which was the internal control and the G and A allele were detected by 110 bp and 235 bp fragments, respectively.

The sequences of the primers were:

G-For: 5'-AGGATAGAGAAAATAATAGAAAACATT-3'
G-Rev: 5'-AACCCATAATAAGAAATAAGTTTTTAC-3'
A-For: 5'-TCCTGAGTAAACTAGGTCACAA-3'
A-Rev: 5'-ACGTTAAGTTCAGAAAGATTAATTC-3'

PCR was carried out using gDNA isolated from buffy coat taken from each participant using the Invitrogen Purelink Genomic DNA kit as described in section 2.2.1. The PCR was carried out in a 10 µl total volume with 1 µl of 0.8 µM-1.2 µM each of G-For and A-Rev and 1 µl of 1.2 µM each of G-Rev and A-For, 5 µl of GoTaq® Green Master Mix (Promega) and 1 µl to 4.5 µl of gDNA depending on its concentration that varied from 7.2 to 61 ng/µl. For a few samples, the reaction volume had to be doubled to 20 µl to get

enough PCR product to be visible. Thermocycling was performed in a Techne TC-512 machine with an initial denaturation at 96^{o}C for 5 min, followed by 30 cycles of denaturation at 94^{o}C for 45 sec, annealing at 56^{o}C for 45 sec and extension at 72^{o}C for 45 sec and a final extension at 72^{o}C for 5 min. PCR products were separated on a 2.5% agarose gel using 0.5X TBE buffer system at 120 V for 1 hour.

2.2.5. Statistical measurements

Genotype frequencies for five SNPs were examined for compliance with Hardy-Weinberg (HW) equilibrium using the equation $p^2+q^2+2pq= 1$ where p and q are frequencies of each allele for each SNP. Chi square (χ^2) analysis for the whole group of 239 particpants was done to compare observed (O) with expected (E) values using the equation $\chi^2= \sum (O-E)^2/E$

O= observed values
E= expected values

2.3. Results

2.3.1. Genotyping and haplotyping of *Bsm*I, *Apa*I and *Taq*I SNPs

Several studies have reported the PCR analysis of the *Bsm*I, *Apa*I and *Taq*I SNPs individually, with each study reporting different SNP specific primers (Lins et al., 2007; Mitra, Desai, & Khatkhatay, 2006; Rezende et al., 2007; Uitterlinden et al., 1996) (Figure 2.5). None of these studies could provide information regarding haplotypes. The study of Uitterlinden et al. (1996) described the amplification of the whole locus with subsequent restriction enzyme digestion that could identify what combinations of alleles each individual had. For this reason the primers F^{11} and R^{11} as shown in Figure 2.5 were chosen for this study. These primers are called VDR1 and VDR4, respectively (Uitterlinden et al., 1996). These primers allowed amplification of a PCR product that included the three SNPs. This product could then be digested with each restriction enzyme separately to identify

each SNP genotype and could be digested with all three enzymes in combination which identified the individual's haplotype for all three SNPs.

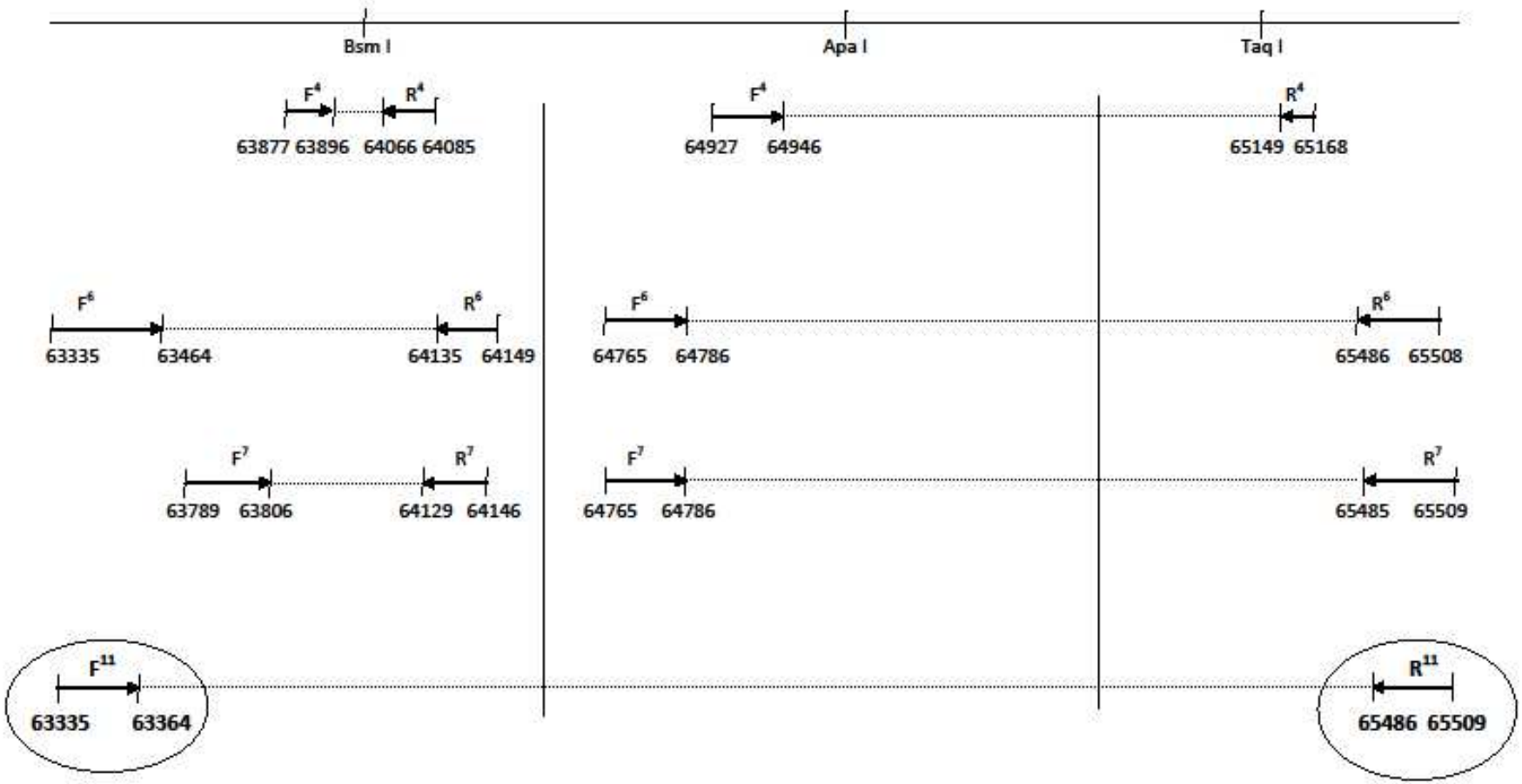


Figure 2.5. A schematic representation of the relative position of primers that were studied for detecting the *Bsm*I, *Apa*I and *Taq*I SNPs. These relative positions of primers were obtained as a result of BLAST (*Basic Local Alignment Search Tool*) between primer sequences from various studies with the human genome. F-forward primer; R-reverse primer; [4]- primers from Lins et al. (2007); [6]-primers from Rezende et al. (2007); [7]-primers from Mitra et al. (2006); [11]-primers from Uitterlinden et al. (1996). Figure is not drawn to scale.

PCR with VDR1 and VDR4 (adapted from Uitterlinden's study) gave a product of the expected size of 2229 bp (Figure 2.6A). However, there were a few non-specific and undigested fragments observed while carrying out this procedure. A faint non-specific fragment of approx 4.6 kb was consistently observed in the PCR with VDR1 and VDR4 primers for all the samples (Figure 2.7). This was not reported by Uitterlinden et al. (1996) and may have been due to a threefold higher concentration of $MgCl_2$ and lower concentration of KCl in GoTaq® Green Master Mix (Promega) as compared to the buffer system used by Uitterlinden et al. for their PCR. Both these factors are well known for causing the formation of non-specific fragments.

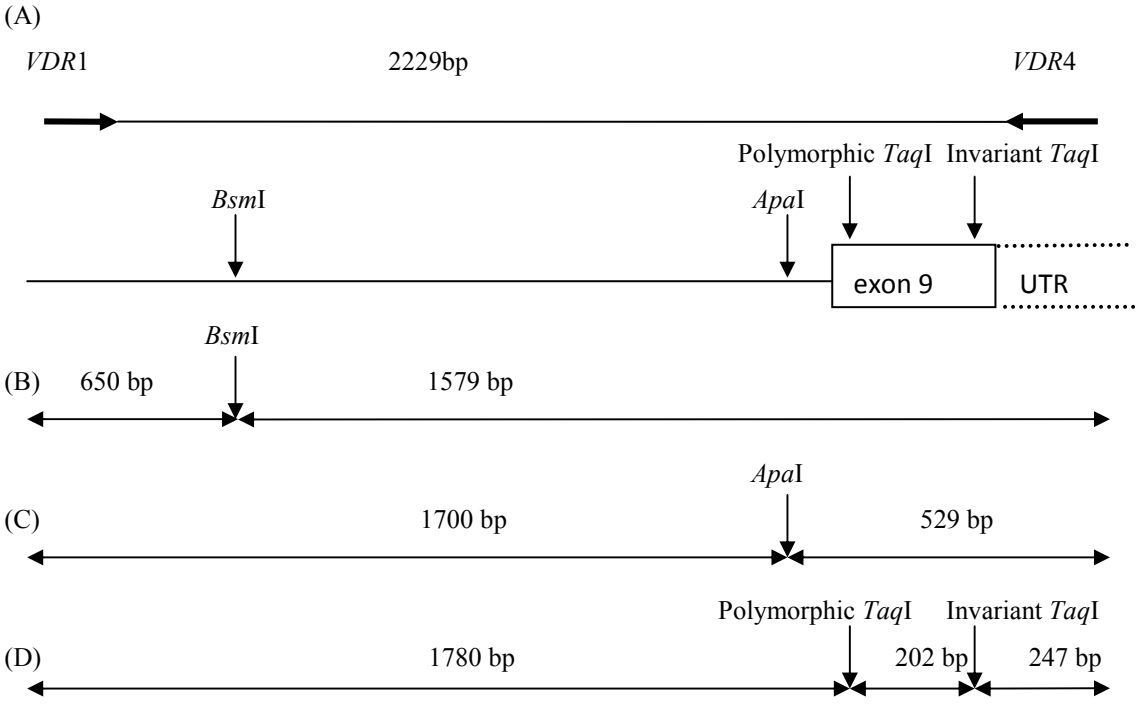


Figure 2.6. A schematic representation of the strategy used for determining genotypes at the 3' end of the *VDR* locus: (A) The *Bsm*I, *Apa*I and *Taq*I sites were amplified as one fragment using the VDR1 and VDR4 primers. The PCR product was digested with the restriction enzyme separately and together and the possible fragment sizes after individual digestion by (B) *Bsm*I, (C) *Apa*I and (D) *Taq*I restriction enzymes are shown. Invariant *Taq*I site was always observed as a result of the presence of an invariant *Taq*I site in exon 9.

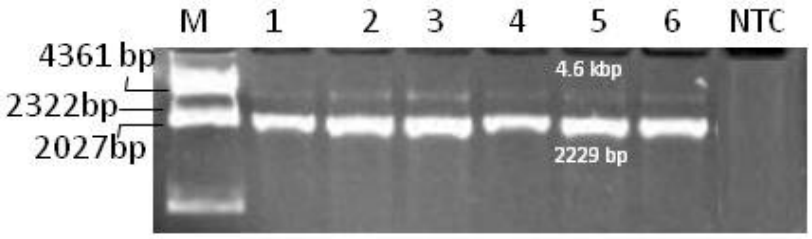


Figure 2.7. Agarose gel electrophoresis (1%, 1X TAE) showing the 2229 bp PCR product and non-specific fragment (approx. 4.6 kbp) as a result of amplification of different gDNA samples (lane 1-6) using the VDR1 and VDR4 primers. Lane M- Lambda *Hind* III; Lane NTC- No template control.

Digestion with each enzyme separately identified the genotype for each SNP. Digestion of the 2229 bp PCR product with *Bsm*I would result in two fragments of 650 bp and 1579 bp (Figure 2.6B). When an individual is homozygous for the presence of the *Bsm*I restriction enzyme site (genotype bb), only these two fragments are expected. For individuals who are homozygous for the absence of the *Bsm*I restriction enzyme site (genotype BB), the PCR product would not be digested so only the 2229 bp would be expected. For those who are heterozygous (genotype Bb), 2229 bp, 1579 bp and 650 bp fragments would be expected (Figure 2.8A).

Digestion of the 2229 bp PCR product with *Apa*I would result in two fragments of 1700 bp and 529 bp (Figure 2.6C). When an individual is homozygous for the presence of the *Apa*I restriction enzyme site (genotype aa), only these two fragments would be expected. For the individuals who are homozygous for the absence of the *Apa*I restriction site (genotype AA), the PCR product would not be digested and therefore, the only fragment expected would be 2229 bp. For those who are heterozygous (genotype Aa), the fragments expected were 2229 bp, 1700 bp and 529 bp (Figure 2.8B)

In contrast with *Bsm*I and *Apa*I, digestion of the 2229 bp PCR product with *Taq*I could result in three fragments of 1780 bp, 202 bp and 247 bp ((Figure 2.6D). An invariant *Taq*I site just 3' to the polymorphic *Taq*I site would be present irrespective of the sequence of the polymorphic *Taq*I site, resulting in a 247 bp fragment after digestion (Figure 2.2.). When an individual is homozygous for the presence of the variable *Taq*I restriction enzyme site (genotype tt), only these three fragments would be expected. For individuals who are homozygous for the absence of this *Taq*I restriction enzyme site (genotype TT), the PCR

product would not be digested at the polymorphic site and therefore, the fragments expected would be 1982 bp and 247 bp. For those who are heterozygous (genotype Tt), the fragments expected would be 1982 bp, 1780 bp, 202 bp and 247 bp (Figure 2.8C).

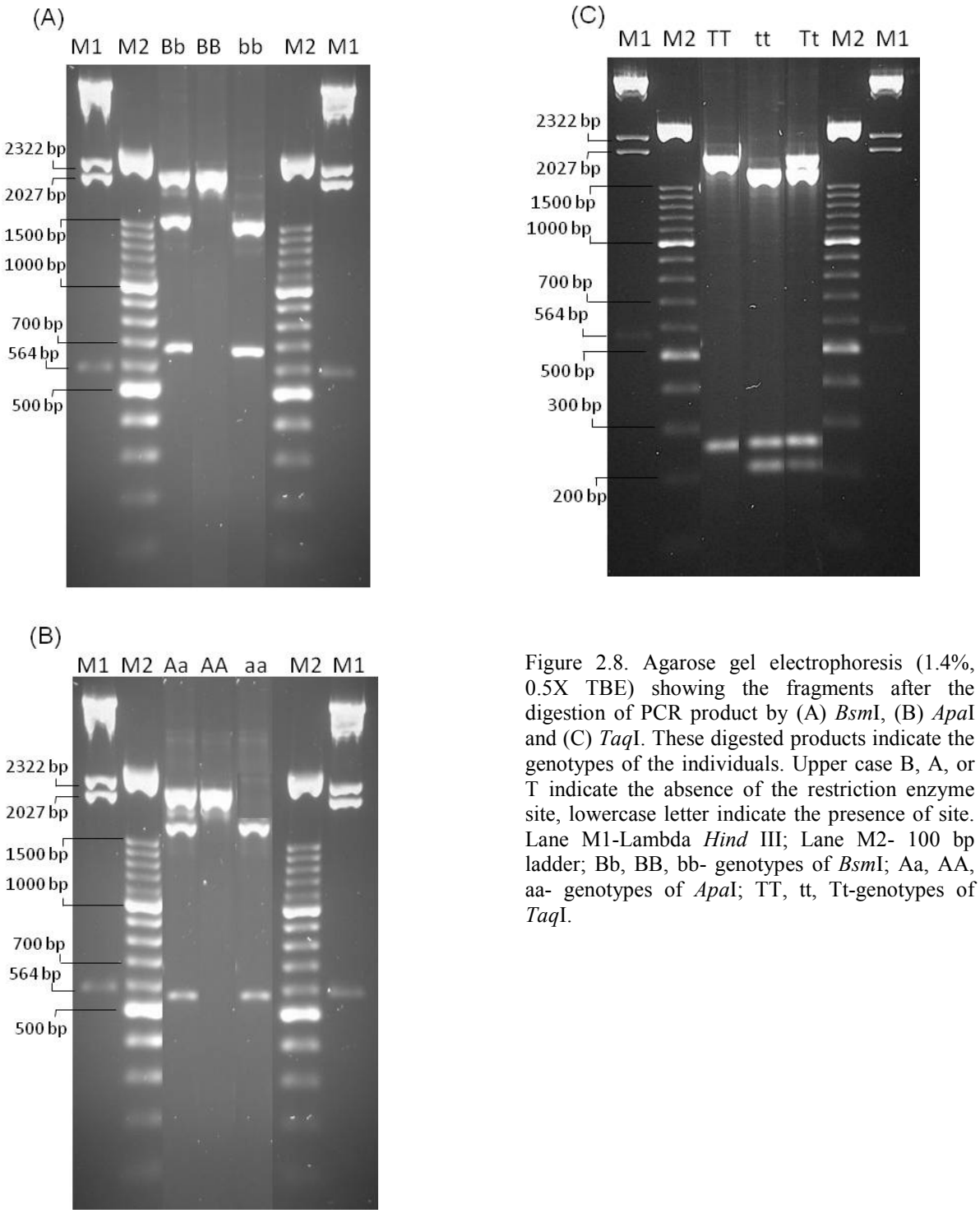


Figure 2.8. Agarose gel electrophoresis (1.4%, 0.5X TBE) showing the fragments after the digestion of PCR product by (A) *Bsm*I, (B) *Apa*I and (C) *Taq*I. These digested products indicate the genotypes of the individuals. Upper case B, A, or T indicate the absence of the restriction enzyme site, lowercase letter indicate the presence of site. Lane M1-Lambda *Hind* III; Lane M2- 100 bp ladder; Bb, BB, bb- genotypes of *Bsm*I; Aa, AA, aa- genotypes of *Apa*I; TT, tt, Tt-genotypes of *Taq*I.

A summary of the expected sizes of fragments after digestion with *Bsm*I, *Apa*I and *Taq*I restriction enzymes separately is shown in Table 2.2.

Table 2.2. Expected size fragments after digestion of PCR products by *Bsm*I, *Apa*I and *Taq*I restriction enzymes individually

SNP	*Genotype*	*Sizes of RFLP-PCR fragments*
*Bsm*I	BB	2229 bp
	Bb	1579 bp, 650 bp
	bb	2229 bp, 1579 bp, 650 bp
*Apa*I	AA	2229 bp
	Aa	2229 bp, 1700 bp, 529 bp
	aa	1700 bp, 529 bp
*Taq*I	TT	1982 bp and 247 bp
	Tt	1982 bp, 1780 bp, 202 bp, 247 bp
	tt	1780 bp, 202 bp, 247 bp

The genotypes for these three SNPs were in HW equilibrium for this cohort of 239 participants which meant that the frequencies of genotypes of all three SNPs were fairly constant in this population (P>0.05) (Table 2.3). So far, all studies conducted on these SNPs in different ethnic populations have reported that the genotypes are in HW equilibrium (Bid et al., 2005; Vupputuri et al., 2006). Therefore, like other populations, the genotype and allele frequencies have also remained constant for this population and have not been influenced by mutations, genetic drift etc. Since these three SNPs were in HW equilibrium, it is likely that there were no errors in the genotyping method adapted for all of them and that the genotyping method yielded the results that fit the expected number under HW equilibrium.

Table 2.3. χ^2 values of the three SNPs of VDR gene

SNP	χ^2 *value**
*Bsm*I	0.02
*Apa*I	1.16
*Taq*I	0.004

**P*>0.05

The frequencies of the genotypes of each SNP were determined and compared with the published data which reported for the individuals from the Indian subcontinent (Bid et al., 2005; Jafar et al., 2009; Mitra et al., 2006; Vupputuri et al., 2006) (Table 2.4). This study showed that the *Bsm*I Bb, *Apa*I Aa and *Taq*I TT genotypes were the most common in this cohort of 239 women. Even though other studies used for comparison focussed on populations from Indian subcontinent, the percentages of individuals with most of the genotypes of the SNPs had a wide range. For example, the percentage of individuals with a *Taq* Tt genotype in Vupputuri et al. (2006) was 85.4% whereas the percentage for the same genotype reported by Bid et al., (2005) was 40% and lowest percentage was reported in the study of Mitra et al. (2006) (35%).

This study showed that for the *Bsm*I SNP, the most common genotype was Bb (49.8%). This was also found in the populations studied by Mitra et al. and Vupputuri et al. The least common genotype for this study was the BB (19.3%) which was also found by Vupputuri et al, but not by Mitra et al. In Mitra's study, the least common genotype was bb (28.5%) but BB and bb genotypes were present in close to equal numbers which could be due to random nature of participant selection.

For *Apa*I SNP, this study showed that Aa (46%) was the most common genotype which was also found by Bid et al. (44%). Mitra et al. found that the frequencies of Aa along with AA genotypes (37%) were higher than aa genotype. The least common genotype for the present study was the aa (21.8%) which was also found by Bid et al. (20%) and Mitra et al. (26%).

This study found TT of *Taq*I SNP the most common genotype (47.3%). This was found in populations studied by Bid et al., Mitra et al. but not by Vupputuri et al. The latter study found Tt (85.4%) as the most common genotype which could be due to extremely small sample size which was studied (n=48). However, the least common genotype tt was the same for all studies (present study- 9.7%; Bid et al.- 11%; Mitra et al.- 29.2%; Vupputuri et al.- 6.3%).

Table 2.4. Comparison of genotype frequencies of *Bsm*I, *Apa*I and *Taq*I in this study and various other studies on Indian women. Figures in **bold** show the most common genotype in that particular study.

SNP		*This study (%)*	*Bid et al., (2005) (%)*	*Mitra et al., 2006 (%)*	*Vupputuri et al., 2006 (%)*
*Bsm*I	BB	46 (19.3%)	–	79 (32.1%)	8 (16.7%)
	Bb	**119 (49.8%)**	–	**97 (39.4%)**	**21 (43.7%)**
	bb	74 (30.9%)	–	70 (28.5%)	19 (39.6%)
Total		239	–	246	48
*Apa*I	AA	77 (32.2%)	54 (36%)	**91 (37%)**	–
	Aa	**110 (46%)**	**66 (44%)**	**91 (37%)**	–
	aa	52 (21.8%)	30 (20%)	64 (26%)	–
Total		239	150	246	–
*Taq*I	TT	**113 (47.3%)**	**170 (49%)**	**88 (35.8%)**	4 (8.3%)
	Tt	103 (43.1%)	138 (40%)	86 (35%)	**41 (85.4%)**
	tt	23 (9.6%)	38 (11%)	72 (29.2%)	3 (6.3%)
Total		239	346	246	48

The combination of *Bsm*I, *Apa*I and *Taq*I SNPs (haplotype) carried by each of the 239 individuals was also determined as was the frequency of each identified haplotype. Five different haplotypes at the *VDR* gene locus were identified and coded 1 to 5 (Figure 2.9). These haplotypes combine to give eleven genotype combinations in this population, the frequencies of which are shown in Table 2.5. Haplotype allele refers to the five possible allelic combinations for the three SNPs that exist in this population while haplotype genotype refers to the eleven combinations of these haplotype alleles that were shown to occur in these individuals. In other words, the term 'haplotype allele' refers to the haplotype on one chromosome and 'haplotype genotype' refers to both chromosomal haplotypes that an individual possesses. The five haplotype alleles found in this population were bAT, BAt, bAT, BAT and bAt where the uppercase letter denoted absence for that restriction enzyme site; B or b for *Bsm*I SNP; A or a for *Apa*I SNP; T or t for *Taq*I SNP. Each individual would have two haplotype alleles in their genome, one on each chromosome. Which two haplotype alleles they have, comprises their haplotype genotype.

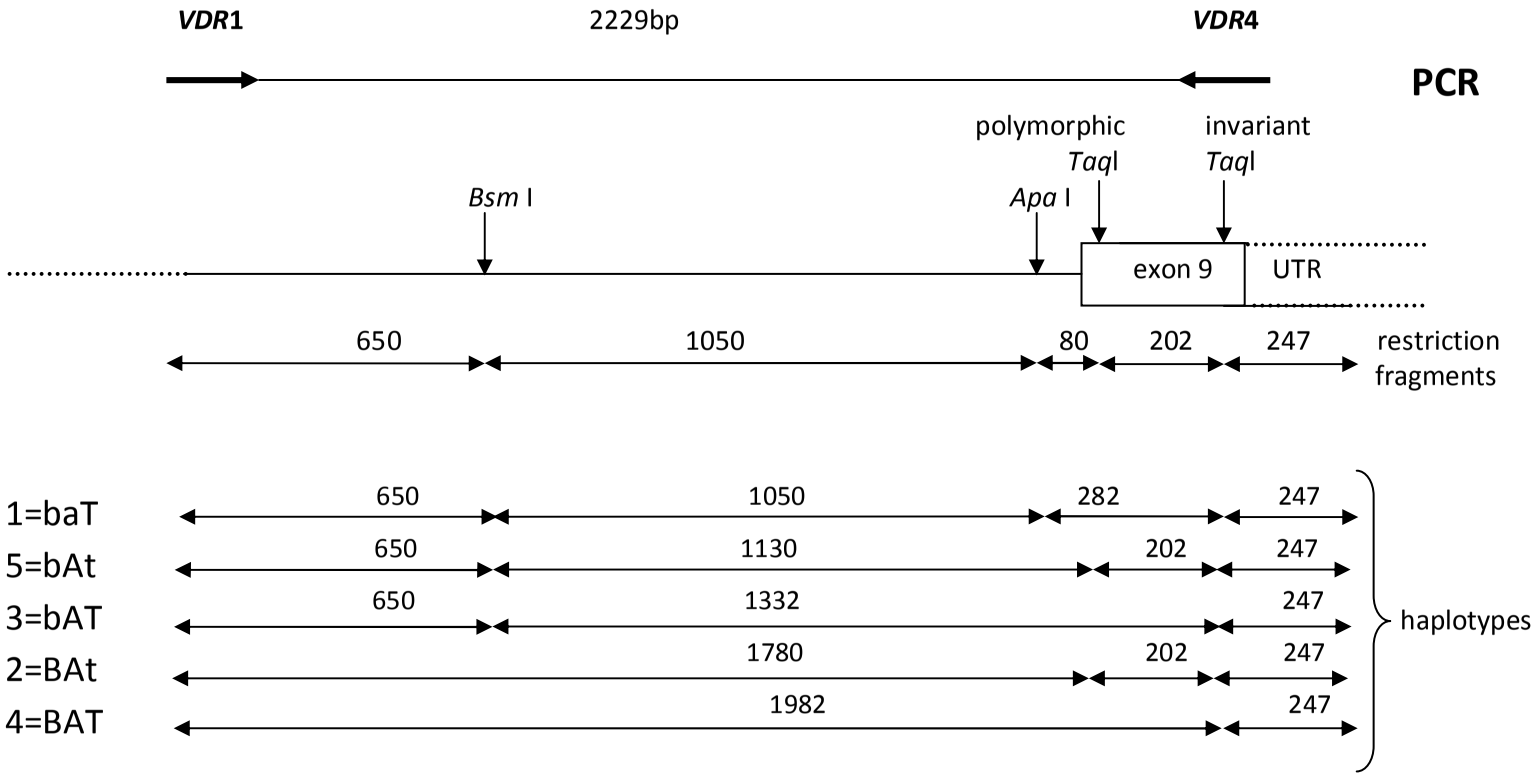


Figure 2.9. Schematic representation of the strategy used for determining haplotypes at the 3'end of the *VDR* locus: The *Bsm*I, *Apa*I and *Taq*I sites were amplified as one fragment using the VDR1 and VDR4 primers. The PCR product was digested with the restriction enzyme separately and together and the possible fragment sizes after combined digestion are illustrated. Uppercase B, A, or T indicates the absence of the restriction enzyme site, lowercase indicates the presence of the restriction enzyme site. The 650 bp, 1050 bp, 80 bp, 202 bp and 247 bp fragments will appear for those haplotypes carrying a *Bsm*I, *Apa*I and *Taq*I recognition sites. Invariant *Taq*I sites were always observed as a result of the presence of an invariant *Taq*I site in exon 9. Figure is not drawn to scale.

Table 2.5. Comparison of the frequencies of haplotype on one chromosome (haplotype allele) and on two chromosomes (haplotype genotype) between this study and Uitterlinden et al. (1996).

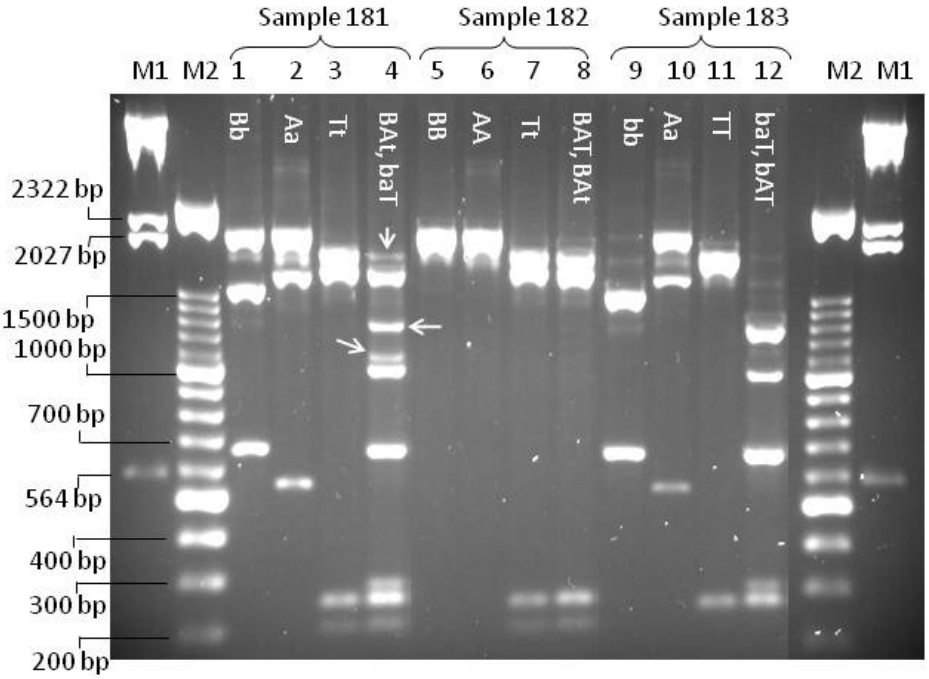
	Code	*Frequency (%)*	
		Uitterlinden et al., 1996	This study
Haplotypes[+]			
baT	1	869 (48.2%)	214 (44.8%)
BAt	2	730 (40.5%)	148 (30.9%)
bAT	3	191 (10.5%)	52 (10.9%)
BAT	4	10 (0.6%)	63 (13.2%)
bAt	5	4 (0.2%)	1 (0.2%)
Total		1804 (100%)	478 (100%)
Genotypes[*]			
BAt-baT	[2,1]	331 (37%)	64 (26.8%)
baT-baT	[1,1]	212 (25%)	52 (21.8%)
baT-BAT	[1,4]	2 (0.2%)	26 (10.9%)
BAt-BAt	[2,2]	155 (17%)	22 (9.1%)
bAT-baT	[3,1]	88 (9.7%)	20 (8.4%)
BAt-bAT	[2,3]	79 (8.6%)	20 (8.4%)
BAt-BAT	[2,4]	8 (0.9%)	19 (7.9%)
BAT-bAT	[4,3]	-	8 (3.4%)
BAT-BAT	[4,4]	-	5 (2.1%)
bAT-bAT	[3,3]	12 (1.2%)	2 (0.9%)
BAt-bAt	[2,5]	2 (0.2%)	1 (0.3%)
baT-bAt	[1,5]	2 (0.2%)	-
Total		902 (100%)	239 (100%)

[+] = number of chromosomes
[*] = number of individuals

The haplotype of each individual were identified by digestion of the 2229 bp PCR product with all three restriction enzymes together. The haplotypes were determined by the pattern of restriction fragments as illustrated in Figure 2.9. For example, the fragment of 650 bp would only appear for those haplotypes carrying a *Bsm*I recognition site, the fragment of 202 bp would appear only for those haplotypes carrying a *Taq*I recognition site, while the aT fragment would only appear for those haplotypes carrying an *Apa*I recognition site but

no *Taq*I recognition site (Figures 2.9, 2.10). Similarly, a 1050 bp fragment corresponded to the presence of both *Bsm*I and *Apa*I recognition sites. Based on the combination of presence or absence of restriction enzyme sites for the three SNPs, when an individual had a baT haplotype allele, 650 bp, 1050 bp, 282 bp and 247 bp fragments were expected as the presence of the *Bsm*I and *Apa*I sites would produce 650 bp and 1050 bp fragments but absence of the *Taq*I site would produce a 282 bp fragment along with the fragment produced by the invariant site of *Taq*I (247 bp) (Figures 2.9, 2.10). When an individual had the bAt haplotype allele, 650 bp, 1130 bp, 202 bp and 247 bp fragments were expected as the presence of *Bsm*I and *Taq*I sites would produce 650 bp and 202 bp fragments but absence of *Apa*I site would produce a 1130 bp fragment along with the invariant 247 bp fragment (Figure 2.9). For the individuals who had the bAT haplotype allele, the PCR product would not be digested with *Apa*I and *Taq*I producing a 1332 bp fragment but the presence of the *Bsm*I site would produce a 650 bp fragment along with the invariant 247 bp fragment (Figures2.9, 2.10). For those who had the BAt haplotype allele, the PCR product would only be digested with *Taq*I to produce a 202 bp fragment and absence of *Bsm*I and *Apa*I sites would produce a 1780 bp fragment along with the invariant 247 bp (Figures 2.9, 2.10). In case of absence of any site, the BAT haplotype allele, only a 1982 bp fragment would be expected along with the 247 bp fragment (Figures 2.9, 2.10).

(A)



(B)

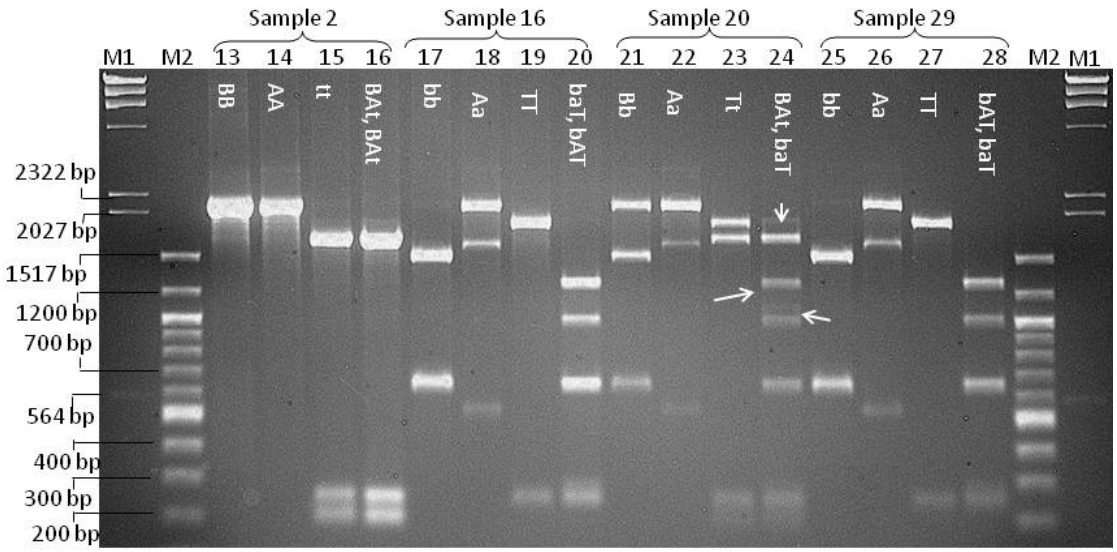


Figure 2.10. Gel electrophoretic separation pattern of RFLP-PCR products in (3A) 0.5XTBE and (3B) 1XTAE buffer systems electrophoresed at 35V for 8 hours and 75V for 2.5 hours, respectively. Lane M1-Lambda *Hind* III; lane M2-100 bp ladder; lane 1,5,9,13,17,21,25,29- PCR product digested with *Bsm*I; lane 2,6,10,14,18,22,26,30-PCR product digested with *Apa*I; lane 3,7,11,15,19,23,27,31-PCR product digested with *Taq*I; lane 4,8,12,16,20,24,28,32- PCR product digested with *Bsm*I, *Apa*I and *Taq*I. The white arrows show the undigested fragments (1982 bp, 1332 bp and 1130 bp) by *Apa*I and *Taq*I in samples 181 and 20.

To identify the haplotype unambiguously, it was not adequate to analyse only the combined digests. Incomplete digestion was consistently observed in spite of doubling the concentration of each enzyme and increasing the time for digestion (Figure 2.10). Each sample had to be digested with each enzyme separately and the fragment patterns were compared (Figure 2.10). Incomplete digestion was particularly evident in the haplotype BAt-baT (Figure 2.10, lanes 4 and 24 corresponding to samples 181 and 20, respectively) where the presence of 1982 bp, 1332bp, 1130 bp fragments were the result of incomplete digestion by *Taq*I and *Apa*I. Various steps were taken to ensure complete digestion such as increasing digestion time from 30 min to 45 min for each enzyme individually and in combination. Also, the number of units for each enzyme was increased to 5U in the combined digestions but it did not eliminate incomplete digestion products. No difference was observed from that seen with half the amount of enzyme in combined digestion. However, the use of the increased amount of *Apa*I (10U) in the individual digestion as compared to *Bsm*I and *Taq*I (1.25U) was due to incomplete digestion of the PCR product by *Apa*I. Many samples initially appeared to be heterozygous for the *Apa*I site (Aa) with 1.25U. However, HW analysis of these indicated a genotyping error. An increased amount of *Apa*I was then tested and found to be appropriate for correct genotyping. Single enzyme digestions revealed the genotype of each individual and the presence of incomplete digestion products in the combined digestion could be resolved by reference to the genotype data. Without the fragment pattern from the individual digests, it would have been impossible to assign a haplotype unambiguously. The genotypes of three samples heterozygous and homozygous for the three sites were confirmed by sequencing and the results obtained were as expected. This showed that the genotyping technique, RFLP-PCR, was very reliable.

Interestingly, the 80 bp band which appears when *Apa*I and *Taq*I recognition sites are present (at genotype) was never observed using either the TAE or TBE buffer systems. Various papers have reported that the haplotype Bat and bat are very rare and this could be the reason for the absence of ‘at’ in this group.

The report of Uitterlinden et al., (1996) was used as the basis for the genotyping/haplotyping technique. This was the only paper that appears to have been

published describing the frequencies of the five different haplotype variants and their combinations leading to 10 genotypes as opposed to the 11 found in this study. Table 2.5 compares the haplotype alleles and genotype frequencies between the two studies. This study showed that baT (44.8%) was the most common haplotype allele followed by BAt (30.9%). This was also found in the population studied by Uitterlinden et al. The least common haplotype allele for both the studies was bAt with the same percentage of 0.2%. However, the third most common haplotype allele for both studies differed. It was bAT (10.5%) followed by BAT (0.6%) in Uitterlinden's study and BAT (13.2%) followed by bAT (10.9%) in this study. The percentages of bAT for both studies were very close but there was a large difference between the percentages of BAT.

Uitterlinden et al. mentions the existence of 10 combinations of haplotype alleles as opposed to 11 found in this study. Two of the haplotype alleles which were not found in Uitterlinden's study were BAT-BAT and BAT-bAT and one haplotype allele missing in this study was baT-bAt. The frequencies of all the haplotypes and genotypes differed significantly between the two studies. However, the most common haplotype genotype found in both the studies were BAt-baT (this study- 26.8%; Uitterlinden et al.-37%) followed by baT-baT (this study- 21.8%; Uitterlinden et al.-25%). Rest of the haplotype genotypes did not have the same order with respect to decreasing frequencies. The percentages of bAT-baT, BAt-bAT, bAT-bAT and BAt-bAt were very close in both the studies. However, the percentages of baT-BAT, BAt-BAt and BAt-BAT widely differed. Thus, even though the percentages of few haplotype genotypes were very close, they did not follow the same order of occurrence as they had wide range of percentages in both studies.

An interesting aspect of these experiments was the buffer system used for electrophoresis to analyse digested products. Initially, the 0.5X TBE buffer system was used for gel electrophoresis at 35V for 8 hours, documenting the separation patterns under UV-illumination after every 4 hours (Figure 2.10A). Since it was taking a long time to run the gels, and there were 239 genotypes and haplotypes to determine, the 1X TAE buffer system was tested (Figure 2.10B). The TAE buffer system is noted for resolving higher molecular weight bands than TBE which is better at resolving lower molecular weight bands. Figure

2.10A shows that in the TBE buffer, smaller bands are better resolved compared to larger fragments while Figure 2.10B shows that the reverse is true for TAE. Therefore, neither buffer system was completely ideal as the spread of fragment sizes was large. How the haplotypes were determined differed depending on the buffer system. If the TBE buffer system was used then haplotypes were determined by analysing fragments ≤1000 bp after 8 hours. If the TAE buffer system was used, then haplotypes were determined by analysing fragments >1000 bp after 2.5 hours. Since haplotypes could be determined with either system, the TAE buffer system was used to improve throughput as it took less time than gels electrophoresed in TBE.

2.3.2. Genotyping of *Fok*I SNP

Several different primers have been used in other studies to amplify the *Fok*I region of the *VDR* gene (John et al., 2005; Lins et al. 2007; MacDonald et al., 2007; Mitra et al., 2006; Rezende at al., 2007). The relative positions of these primers are shown in Figure 2.11. Primers reported by Rezende et al. were chosen because the fragments generated after digestion (74 bp and 198 bp) by the restriction enzyme, *Fok*I, could be easily resolved by electrophoresis. Other reported primers that would have generated very small molecular weight fragments after digestion which could have been difficult to detect by agarose gel electrophoresis.

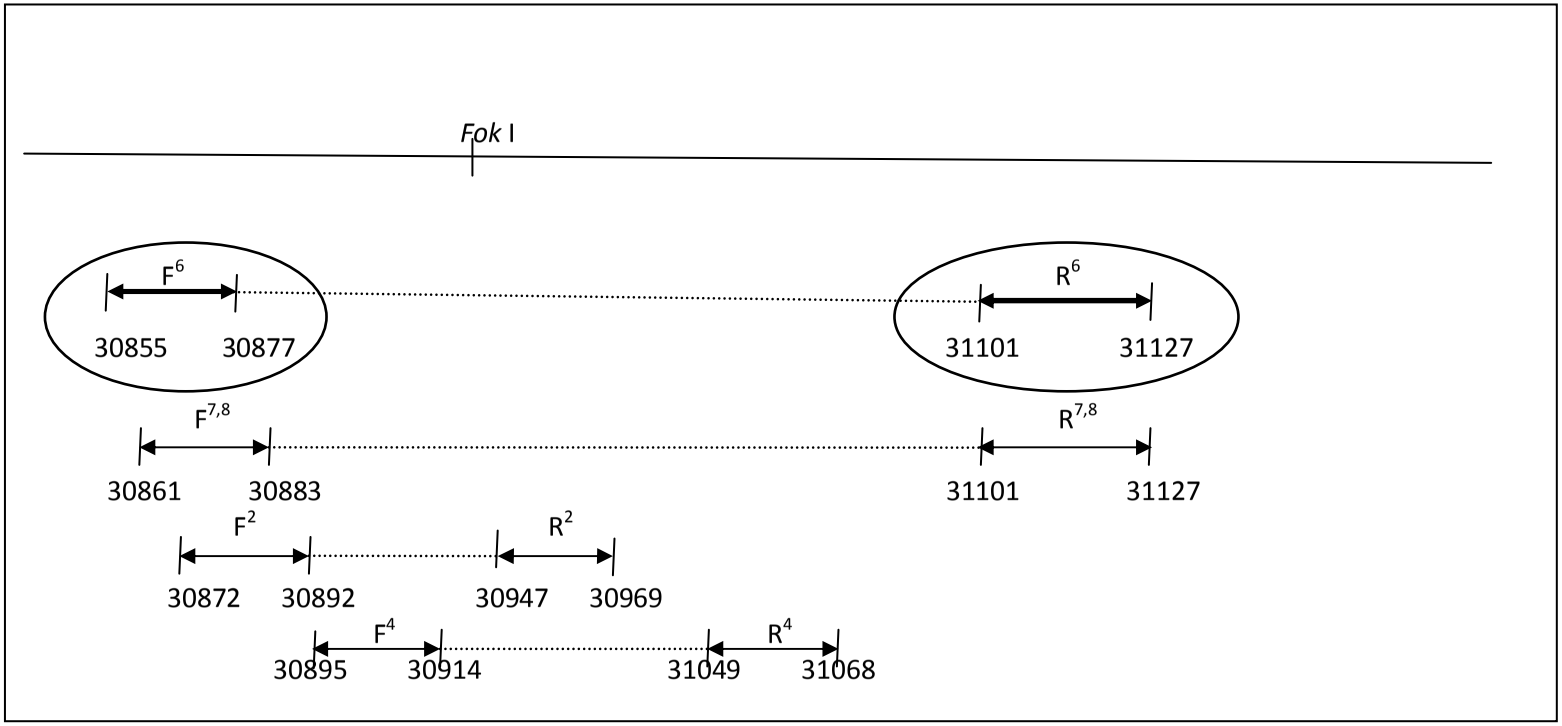


Figure 2.11. Schematic representation of primers that were studied for detecting the SNP site at the *Fok*I site. These relative positions of primers were obtained as a result of BLAST (*Basic Local Alignment Search Tool*) between primer sequences from various studies with the human genome. F-forward primer; R-reverse primer; [2]-primers from John et al. (2005); [4]- primers from Lins et al. (2007); [6]-primers from Rezende et al. (2007); [7]-primers from Mitra et al. (2006); [8]-primers from MacDonald et al. (2007). Figure is not drawn to scale.

The methods of PCR and digestion as used by Rezende et al. (2007) were followed with modifications. The PCR product for *Fok*I using primers described by Rezende et al. yielded a 272 bp fragment (Figure 2.12) which when digested with *Fok*I generated fragments of 74 bp and 198 bp (f allele) (Figure 2.13). Allele F denoted absence of the restriction site, giving a 272 bp band after digestion.

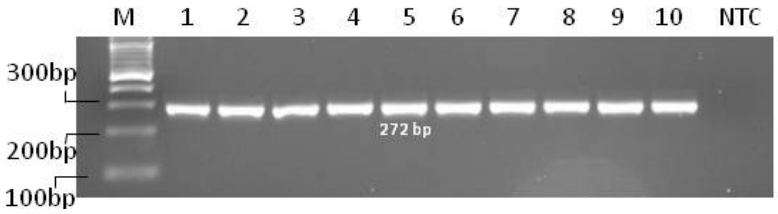


Figure 2.12. Gel electrophoretic separation pattern (2.5%, 0.5X TBE) of 272 bp PCR fragment of different samples (lane 1-10) for *Fok*I digestion. Lane M- 100 bp ladder; Lane NTC- No negative control

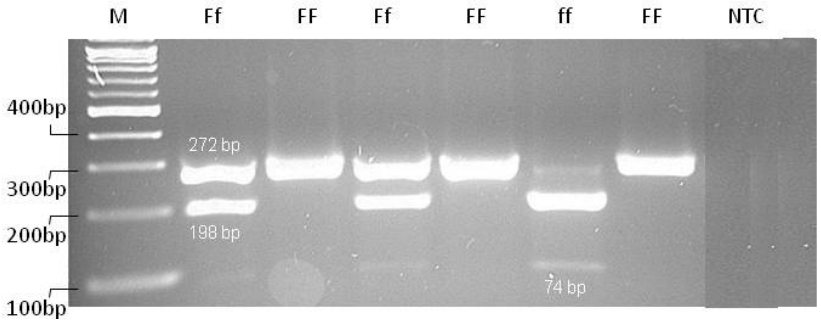


Figure 2.13. Gel electrophoretic separation pattern (2.5%, 0.5X TBE) after *Fok*I digestion of 272bp PCR product. Lane M- 100 bp ladder; FF, Ff, ff-genotypes of *Fok*I; lane NTC- No template control.

Initially, PCR amplifications using the conditions described by Rezende et al. resulted in no detectable amplicons. Therefore, a gradient PCR was undertaken to determine the optimum annealing temperature. This was found to be 63^{o}C. Also, the 25 µl total reaction volume used by Rezende et al. was reduced to 15 µl which yielded the same results and proved to be more cost-effective. To determine the optimum volume/units of enzyme for digestion, the digestion was tested using different amounts of enzyme (10U and 20U) and length of incubation (3 hours and overnight). One µl of enzyme (10U/µl) with overnight digestion was found to be optimal.

Like *Bsm*I, *Apa*I and *Taq*I the genotypes and allele frequencies were in HW equilibrium. The genotypes and alleles for *Fok*I were in HW equilibrium for the cohort of 239 participants (χ^2 value= 1.432; df= 1; P >0.05). This meant that like *Bsm*I, *Apa*I and *Taq*I the frequencies of genotypes have remained constant from generation to generation. This also showed that there were no errors in the genotyping method adapted for this SNP and that the genotyping method yielded the expected results.

Table 2.6 shows the percentages of the genotypes in all 239 individuals. Like *Bsm*I, *Apa*I and *Taq*I, these studies were conducted on Indian women but the genotype with highest percentage differed from study to study. The most common genotype of *Fok*I SNP was FF (58.2%) followed by Ff (34.3%) in this study as well as in the populations studied by Mitra et al. and Vupputuri et al. However, the most common genotype found by Bid et al. and Jafar et al. was Ff followed by FF genotype. Nevertheless, frequencies of Ff and ff genotypes were very close and ff genotype had the lowest frequency in all the studies.

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Table 2.6. Comparison of genotype frequencies of *Fok*I in this study and various other studies among Indian women. Figures in **bold** show the most common genotype in that particular study

Study	*This study*	*Bid et al., 2005*	*Mitra et al., 2006*	*Jafar et al., 2009*	*Vupputuri et al. , 2006*
Genotype					
FF	**139 (58.2%)**	152 (43.9%)	**97 (39.4%)**	270 (47.5%)	**28 (58.3%)**
Ff	82 (34.3%)	**170 (49.1%)**	84 (34.2%)	**284 (49.9%)**	18 (37.5%)
ff	18 (7.5%)	24 (6.9%)	65 (26.4%)	15 (2.6%)	2 (4.2%)
Total	239	346	246	569	48

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2.3.3. Genotyping of Cdx-2 SNP

Several different primers have been used in other studies to amplify the Cdx-2 region of the *VDR* gene (Balcolm et al., 2008; Fang et al., 2003; John et al., 2005; Lins et al., 2007; MacDonald et al., 2006). The relative positions of these primers are shown in Figure 2.14. It was decided for this study to use the approach described by Fang et al. (2003) for two reasons. Firstly, since Cdx-2 is not a restriction site, the RFLP-PCR technique could not be used. Therefore, some of the studies sequenced the region to reveal the genotype at the Cdx-2 site. However, this is very expensive. Since the method of Fang et al. was a cheaper alternative method to determine the genotype based on allele specific multiplex polymerase chain reaction (ASM-PCR), this method was selected. Secondly, most of the previous research aimed at amplification of Cdx-2 referred to the primers by Fang et al. which showed that the method is very reliable. The ASM-PCR uses two primers that are specific to the SNP site together with two primers that flank the SNP site.

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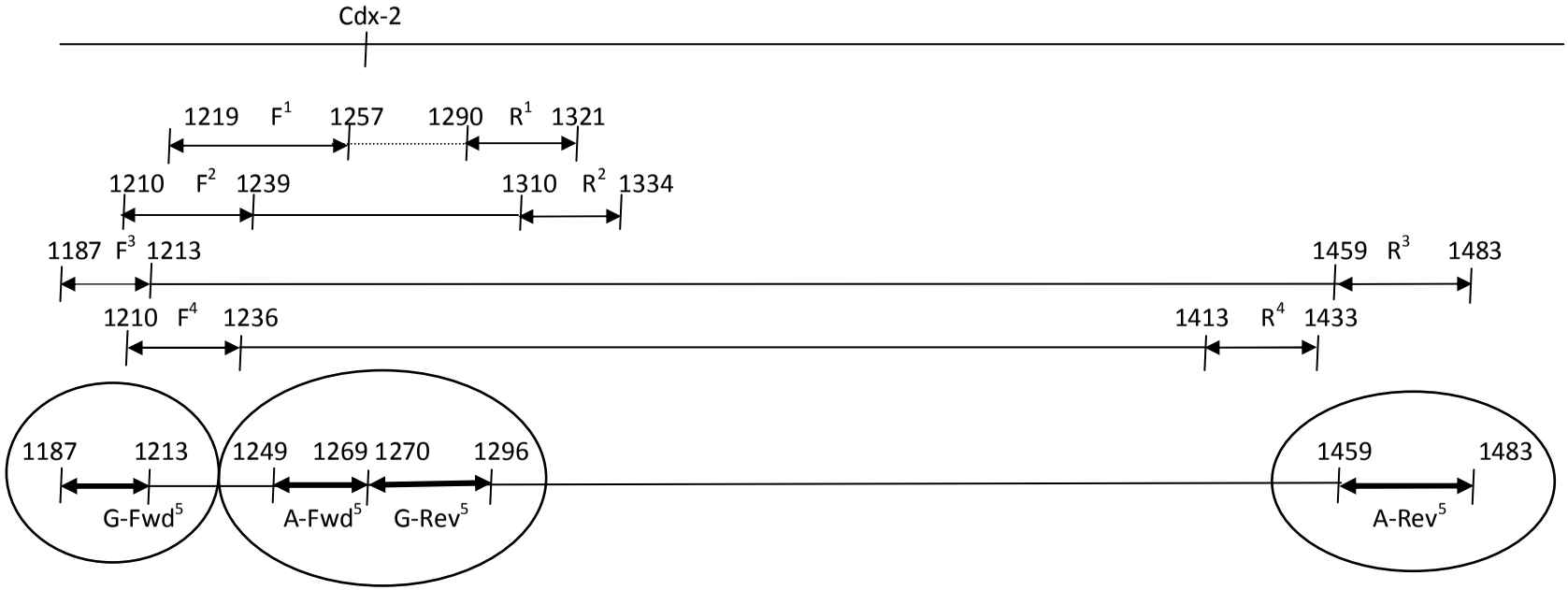

Figure 2.14. A schematic representation of the relative position of primers that have been used for detecting the Cdx-2 SNP site. These relative positions of primers were obtained as a result of BLAST (*Basic Local Alignment Search Tool*) between primer sequences from various studies with the human genome. F-forward primer; R-reverse primer; [1]-primers from Balcom et al.(2008); [2]-primers from John et al. (2005); [3]-primers from MacDonald et al. (2007); [4]-primers from Lins et al. (2007); [5]-primers from Fang et al. (2003). Figure is not drawn to scale.

The external primers G-For and A-Rev generate a 297 bp fragment that serve as an internal control independent of the SNP site. SNP detection is based on the annealing pattern of the internal primers, G-Rev and A-For. The last base of G-Rev is 'C' to match with the G allele of the antisense (-) strand and the last base of A-For is 'A' to match the A allele of the sense (+) strand (Figure 2.15). A 110-bp fragment is the G-allele-specific PCR product which is generated by amplification between the G-For and G-Rev primers and a 235 bp fragment is the A-allele specific fragment which is generated by amplification between the G-For and G-Rev primers (Figures 2.15). Individuals who are heterozygous should yield three fragment, 297bp (internal positive control), 235bp (A allele) and 110 bp (G allele) and those who are homozygotes should yield only two fragments, 297bp with either 235bp or 110bp (Figures 2.16).

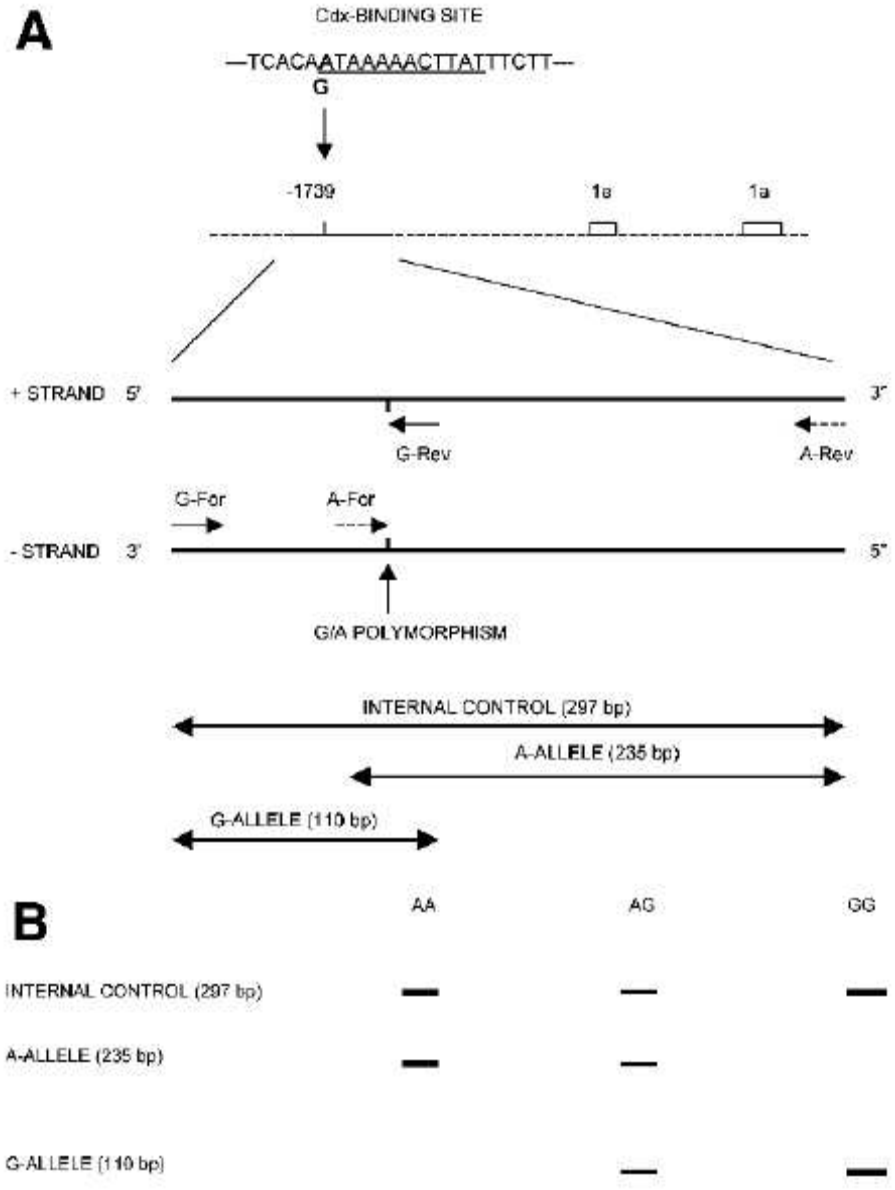

Figure 2.15 (A). Schematic representation of primers for detecting the SNP site at the Cdx-2 site in the *VDR* 1e promoter region. (B) Schematic representation of the expected gel pattern with the PCR product for Cdx-2 genotyping

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From "Cdx-2 polymorphism in the promoter region of the human vitamin D receptor gene determines susceptibility to fracture in the elderly," by Fang et al. 2003, *Journal of Bone and Mineral Research,18*, p.1634. Copyright ©2003 by the American Society for Bone and Mineral Research.

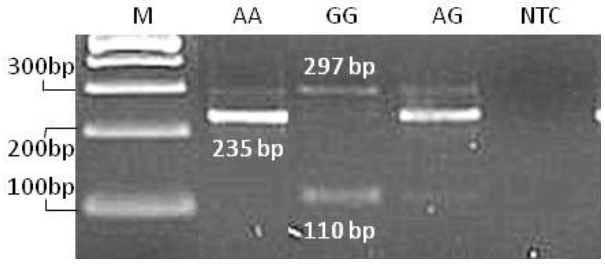


Figure 2.16. Gel electrophoretic separation pattern (2.5%, 0.5X TBE) of ASM-PCR of Cdx-2 products in 0.5X TBE buffer electrophoresed at 120V and visualised after 1 hour. AA, GG and AG-genotypes of Cdx-2 SNP; Lane NTC-no template control.

Unfortunately, the amplification and the cycling conditions as described by Fang et al., had to be modified to yield PCR products routinely. This group used 1 µl of 10X PCR buffer (1X buffer= 10mM Tris-HCl, pH 8.3; 50mM KCl; 1.25mM $MgCl_2$) 0.4 pmol of each G-For and A-Rev, 0.6 pmol of each G-Rev and A-For and 28 cycles of denaturation, annealing and extension. The conditions had to be optimized for the AUT laboratory setting. Increasing the number of cycles from 28 to 30 and doubling the primer concentrations gave a PCR product for most of the samples but all of them showed a very faint 297 bp fragment which was the positive internal control. In order to confirm the latter's presence, primer concentrations of G-For and A-Rev had to be increased from 0.8 µM to 1.2 µM. However, no matter what the conditions were, the internal positive control fragment of 297 bp was always faint and the brightest fragment was the 235 bp fragment.

The genotypes and alleles for Cdx-2 were in HW equilibrium for the cohort of 239 participants (χ^2 value= 3.06; df= 1; P >0.05). This meant that like *Bsm*I, *Apa*I, *Taq*I and *Fok*I both alleles and genotypes of this population were in equilibrium and the frequencies have remained constant from generation to generation. This also showed that there were no errors in the genotyping method adapted for this SNP and that the genotyping method yielded the expected results.

The frequency of the heterozygous genotype AG was the highest followed by the homozygous genotype GG in these 239 women. Table 2.7 shows the percentages of Cdx-2 genotypes of the studies looking at this SNP site. Very few Cdx-2 studies have been carried out on South-Asian women, therefore, data from studies focussing on different ethnicities

were used for comparison (Table 2.7). Most studies along with the present study have reported the AG genotype to be the most common except those of Lurie et al. (2007) and Casado-Diaz (2007) who studied Caucasians and Spanish women, repectively. Lurie et al. and Casado-Diaz found GG genotype with the highest frequency. The least common genotype was the AA in most of the studies except in the population studied by Dickinson et al. (2009). In the latter study, it was GG genotype that was the least common.

Table 2.7. Comparison of genotype frequencies of Cdx-2 in this study and various other studies among different ethnicities. Figures in **bold** show the most common genotype in that particular study.

Study	*This study*	*Selvaraj et al., 2008*	*Dickinson et al., 2009*	*Lurie et al., 2007*		*Casado-Diaz, 2007*
Ethnicity	South-Asian women (%)	South Indian women (%)	Residents of Tasmania (%)	Caucasians (%)	Japanese (%)	Spanish women (%)
Genotype						
AA	43 (18%)	34 (16.5%)	78 (33.2%)	6 (4.1%)	27 (15.8%)	18 (8%)
AG	**132 (55.2%)**	**86 (41.7%)**	**109 (46.4%)**	44 (30.3%)	**99 (57.9%)**	76 (33.8%)
GG	64 (26.8%)	**86 (41.8%)**	48 (20.4%)	**95 (65.5%)**	45 (26.3%)	**131 (58.2%)**
Total	239	206	235	145	171	225

2.4. Discussion

2.4.1. Determination of genotype and haplotype frequencies of *Bsm*I, *Apa*I and *Taq*I SNPs

RFLP-PCR is one of the most common and simple techniques which give results with great confidence for genotyping. It can, however, give rise to inaccurate results if the restriction enzyme digestion is incomplete, thereby resulting in false heterozygotes which was observed in this study for *Apa*I. Nevertheless, these false positives for heterozygotes were identified by comparing the genotype with the haplotype gel patterns and were eliminated by increasing the enzyme concentration. However, for a quick analysis of frequencies of various SNPs, it may be unnecessary and time consuming to create and analyse such haplotype data by gel patterns. Also, the comparison which was carried out in this study between the haplotype and genotype fragment patterns may not always be possible if the SNPs are widely separated on the chromosome as haplotypes with RFLP-PCR can not be observed if SNPs are widely separated. Therefore, it is necessary to know if the observed genotype frequency is in Hardy Weinberg equilibrium. If the genotypes are not in HW equilibrium, then there could be two reasons: either the population is affected by disturbing influences such as mutations, genetic drift etc that result in the predominance of a genotype in that population or appearance of a new allele; or that there are scoring errors resulting in the predominance of heterozygotes and few/none homozygotes. The latter is usually tested before assuming genetic drift as there is a greater possibility of inaccurate genotype calls than disappearance of a homozygote from a population or appearance of a new allele.

RFLP-PCR technique could be used with either buffer system, TAE or TBE, for analysis of the gel fragment pattern. Depending on the fragment sizes to be analysed, either gel systems could be used. For this study, since the fragment pattern after digestion varied from 202 to 2229 bp, haplotypes could be inferred either by analysing the lower molecular weight fragments using TBE buffer system or higher molecular weight fragments using TAE buffer system. Since the electrophoretic separation of DNA in TAE buffer takes less time, 2.5 hours compared to 8 hours for TBE, this study used TAE to analyse haplotypes of 239 women and would recommend this for future work.

Another drawback of RFLP-PCR is the volume of starting material needed to extract DNA which is large. For this study, 50 µl of buffy coat sample was used but it could be reduced to 20 µl. However, the process of extraction is very time consuming especially if there are a large number of samples.

RFLP-PCR helped in determining the frequencies of genotypes and haplotypes at the 3' end of the *VDR* gene. These frequencies differed compared to other studies. The discrepancy in the frequency of the *Taq*I Tt genotype reported in Vupputuri's study, in spite of ethnicity of population being the same as this study, may be due to the small sample size (n=48) which may have given biased and inaccurate results. The disparity between haplotype frequencies reported in this study and that of Uitterlinden et al. (2004) may depend on the ethnicity of the population under study. Uitterlinden's study focussed on a Caucasian population whereas this research studied South-Asian women. By and large, the genotype and haplotype data presented by this study are in agreement with the findings of others with some discrepancies. The major strength of this study was to determine the haplotypes of 239 individuals by RFLP-PCR whereas other studies have used software to 'predict' the haplotypes in a population and so they may not identify a rare haplotype which may exist in the population.

2.4.2. Determination of genotype frequencies of *Fok*I SNP

The method of Rezende et al. (2007) for determination of *Fok*I SNP had a few drawbacks. The incubation temperature reported by them was 55^{o}C for *Fok*I as purchased from Fermentas. The incubation temperature of *Fok*I is actually 37^{o}C whereas Rezende et al. reported using *Fok*I at 55^{o}C. Another enzyme which is an isoschizomer of *Fok*I, known as *Bsg*I, has an incubation temperature of 55^{o}C. It is possible that the authors used *Bsg*I instead of *Fok*I and have reported using the wrong enzyme. Also, the PCR conditions as reported by them had to be optimised for routine use in the AUT lab.

In general, the frequencies of genotypes of *Fok*I SNP found in this study were in agreement with few studies but contrary to few other findings. Lack of similarity in results between the studies focussed on Indian women could be due to sample sizes which differed widely

between the studies. Since environmental conditions such as nutrition can vary greatly from one part of India to another, the lack of similarity between results could also be different due to gene-environment interactions which may have influenced the predominance of a genotype in some groups but not others. Also, since frequencies of FF and Ff were very close, the discrepancies could be due to random nature of participant selection.

2.4.3. Determination of genotype frequencies of Cdx-2 SNP

The PCR for determining Cdx-2 genotype as designed by Fang et al. (2003) included amplification of an internal control. The presence of the internal control fragment was designed to indicate that the PCR worked and amplification conditions were optimal. However, in this study the internal control fragment was always faint. This could have been because of the difference in the concentrations of various components such as KCl and $MgCl_2$ in the GoTaq® Green Master Mix (Promega) compared with the 10X PCR buffer used by Fang et al (2003). Also, the recommended annealing temperature could be more favourable for the 235 bp fragment (A allele) amplification as opposed to either the 297 bp (internal fragment) or the 110 bp (G allele) fragment amplification. However, Fang et al. could successfully amplify all three fragments at the same annealing temperature and did not report any difficulties. To make sure that the internal control was amplified, primer concentrations of the external primers were increased three fold which should have amplified the internal control more efficiently. Only the external primers were increased three fold as these primers were used for amplification of the allele-specific fragment and the internal control whereas the allele-specific primers were increased two fold for efficient amplification of allele-specific fragments. However, this did not make much difference to amplification of all three fragments and the internal control remained faint. Thus, the only possible explanation was the difference in concentration of components in the PCR buffer systems used in the two studies which led to better amplification of the allele-specific product and not the internal control.

Unlike RFLP-PCR where there is amplification of only one fragment which is further digested, ASM-PCR aims at successful amplification of two different alleles and an internal

control at the same temperature, generating three different size fragments. Therefore, the condition for PCR should be such that it does not hinder amplification of any of these fragments and this could be the reason that so many alterations had to be made in ASM-PCR compared to RFLP-PCR. However, unlike RFLP-PCR, this technique is cheaper and quicker as it does not require post-PCR treatment such as restriction enzyme digestion. It amplifies three fragments giving information of the genotype directly after gel electrophoresis of PCR products. It could also be used to determine haplotype of SNPs located within a limited distance by designing allele-specific primers, as described for AS-PCR. It is possible, that in the near future RFLP-PCR is replaced by ASM-PCR for quick genotyping of SNPs.

Similar to *Fok*I, the frequencies of genotypes of Cdx-2 SNP found in this study were in agreement with few studies but contrary to few other findings. The reasons for the discrepancies in genotype frequencies of Cdx-2 SNP between this study and other studies could be because of ethnicity of the populations studied. The frequencies of genotypes may differ between populations of different ethnicities, depending on gene-environment interaction and disease predisposition of the population. Thus, the similar findings of this study regarding the highest genotype frequency with other studies showed that different genotypes may have similar frequencies in few ethnic groups but not the others.

Chapter 3

Comparison of real-time PCR

(qPCR)

and

restriction fragment length polymorphism PCR

(RFLP-PCR)

3.1. Introduction

Chapter 2 described the RFLP-PCR approach that was used to determine the genotypes of 239 individuals at four loci in the *VDR* gene; the fifth locus involved ASM-PCR. These approaches proved successful, but during the course of the research it was decided to evaluate alternative methods for genotyping that had the potential of offering speed and accuracy compared to more time-consuming RFLP-PCR.

Of the alternative genotyping methods, it was decided to examine the efficacy of the TaqMan® genotyping assay (Applied Biosystems, CA, USA). Most of the allelic discrimination strategies have been described in section 2.1.5 and the TaqMan® genotyping assay is described below.

3.1.1. Hybridisation

The hybridisation approach employs two allele specific probes that hybridise and are extended only when they perfectly match the target DNA under optimal conditions (Kim & Misra, 2007; Kwok, 2001). Allelic discrimination can only be achieved when the one-base mismatched probe is able to hybridise to the target sequence (Kim & Misra, 2007) (Figure 3.1). The only challenge is to design allele-specific probes that differ by one nucleotide at the SNP site (Kwok, 2001). The efficacy and robustness of the technique depends on the design of the probe, its location on the target DNA, the SNP position and hybridisation conditions that favour perfect matching of the correct probe to its target SNP (Kim & Misra, 2007).

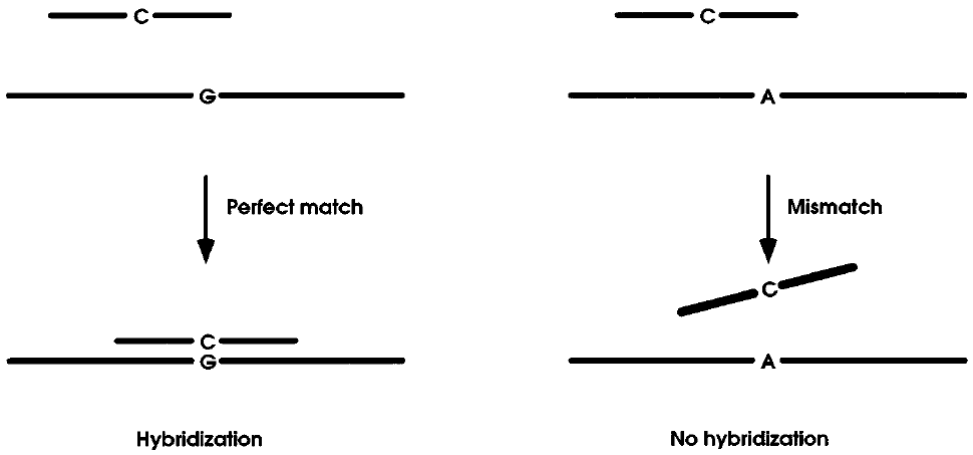


Figure 3.1. Allele specific hybridisation: relies on hybridisation of a perfectly matched probe to the target sequence to achieve allelic discrimination. In case of a mismatched probe under optimal hybridisation conditions, the probe destabilises the hybridisation, thus preventing the product formation.

From "Methods and genotyping single nucleotide polymorphisms ," by Pui-Yan Kwok 2001, *Annual Review of Genomics and Human Genetics, 2*, p.238, 239. Copyright ©2001 by Annual Reviews.

The TaqMan® genotyping assay (Applied Biosystems, CA) employs two allele-specific probes and two common primers that span the SNP site. These probes are fluorescently labelled at one end with a reporter dye such as FAM or VIC and carry a non-fluorescence quencher at the other end. The intact probes do not fluorescence when the reporter and quencher dyes are at close proximity to each other (Holland, Abramson, Watson, & Gelfand, 1991). During the PCR, if there is an exact sequence match, the labelled probe hybridises to its complementary sequence located between the PCR primers. As the upstream primer is extended, the 5'→3' exonuclease activity of the polymerase cleaves the probe releasing the reporter dye from the quencher. Fluorescence then occurs which is detected by the optical system of the PCR machine. If there is a mismatch between the probe and the target sequence, no hybridisation will occur and therefore there will be no cleavage and fluorescence (Livak, 1999) (Figure 3.2).

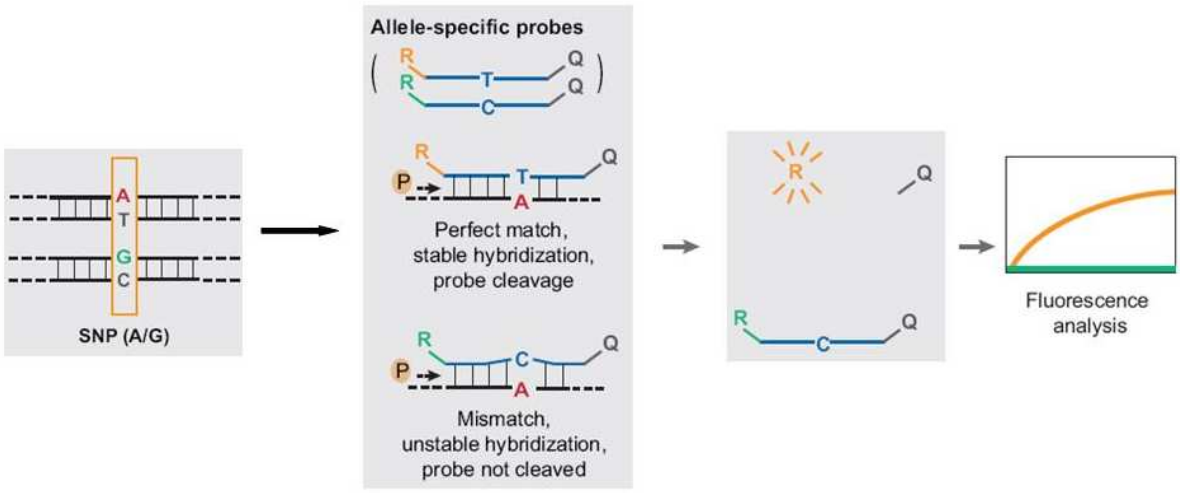


Figure 3.2. The TaqMan® genotyping assay: The assay includes two allele specific probes which are labelled with different dyes at reporter (R) and are non-labelled at the quencher (Q). The perfectly matched probe is cleaved and extended by a polymerase possessing 5'→3'exonuclease activity, thus freeing the reporter from quencher, and the resulting fluorescence is then detected.

Modified from "SNP Genotyping: Technologies and Biomedical Applications," by Sobin Kim and Ashish Misra 2007, *Annual Review of Biomedical Engineering, 9*, p.291. Copyright ©2007 by Annual Reviews.

In view of the above, it was decided to evaluate a TaqMan® assay to determine the alleles at the three polymorphic loci at the 3' end of the *VDR* gene: *Bsm*I, *Apa*I and *Taq*I SNPs.

3.2. Methods and Materials

3.2.1. gDNA extraction for RFLP-PCR

gDNA was isolated from buffy coat taken from each participant and was isolated using the Invitrogen Purelink Genomic kit as described in section 2.2.1.

3.2.2. gDNA extraction for qPCR

The extraction of gDNA for quantitative PCR (qPCR) was performed using the Applied Biosystems TaqMan® Sample-to-SNP™ kit according to the manufacturer's instructions. 2 µl of each buffy coat was added to 20 µl of Lysis Solution and incubated for three minutes

at room temperature. After the incubation, 20 µl of DNA Stabilizing Solution was added and stored at -20^{o}C until further use.

3.2.3. RFLP-PCR genotyping of *Bsm*I, *Apa*I and *Taq*I

gDNA extracted using the Invitrogen Purelink Genomic kit was used for PCR and subsequent digestion with each enzyme, *Bsm*I, *Apa*I and *Taq*I, individually to reveal the genotypes as described in chapter.

3.2.4. qPCR genotyping of *Bsm*I, *Apa*I and *Taq*I

gDNA samples extracted using Applied Biosystems TaqMan® Sample-to-SNP™ kit were genotyped for the *Bsm*I, *Apa*I and *Taq*I SNPs using Applied Biosystems TaqMan genotyping assay kits unique for each SNP. qPCR was carried out in an Eppendorf Mastercycler EP Realplex machine.

PCR was performed using 2 µl of the extracted gDNA, 5 µl of TaqMan GTXpress Master Mix (Applied Biosystems), 0.5 µl of TaqMan genotyping assay mix containing SNP specific primers and probes for either *Bsm*I, *Apa*I or *Taq*I and 2.5 µl of water in a final reaction volume of 10 µl. Each sample was replicated twice in the same qPCR plate and the positive controls were replicated thrice. For *Bsm*I, the positive controls for the genotypes BB, Bb and bb were samples 182, 183 or 70 and 181 respectively; the *Apa*I genotypes AA, aa and Aa corresponded to samples 182, 70 and 181, respectively; and the *Taq*I genotypes TT and tt corresponded to samples 1 and 2, respectively. These positive controls samples were analysed by RFLP-PCR and confirmed by sequencing using an ABI 3100-Avant Genetic Analyzer (Applied Biosystems) using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems). The thermal conditions were exactly the same for all three SNPs, as recommended by the Applied Biosystems, which included an initial denaturation at 95^{o}C for 10 min and then 40 cycles of 92^{o}C 15 sec and 60^{o}C for 1 min. Initially, the PCRs for *Taq*I were performed using Applied Biosystem MicroAmp™ Optical 96-well reaction

plates with Applied Biosystem Optical Adhesive Film. However, the weak VIC signal necessitated using Eppendorf twin.tec real-time PCR plates and the same adhesive films that were used for the *Apa*I and *Bsm*I assays.

Data analysis for qPCR was done using the software provided by the Eppendorf *realplex* software v.2.2.The results were analysed by establishing the threshold values for the positive controls. Any fluorescence of VIC and FAM that were detected above or below the threshold values were considered to represent positive or negative allele calls, respectively.

3.3. Results

3.3.1. RFLP-PCR results

Results of the genotype determination by RFLP-PCR are described in section 2.3.1.

3.3.2. qPCR results

Allelic discrimination by TaqMan® genotyping assay relies on perfect matching of the correct probe to its target SNP leading to hybridisation and detection of fluorescence of the respective dye at a particular temperature. However, this technique failed completely using the recommended protocol of Applied Biosystems for their ready madeTaqman assays designed for genotyping the 3'end of the VDR gene. The templates used in these assays were gDNAs extracted from buffy coat samples with the Applied Biosystems' Sample to SNP extraction kit using the protocol as directed. As a result, when the data obtained from RFLP-PCR were compared with qPCR, a lot of discrepancy in the frequencies of homozygotes and heterozygotes for all three SNPs was observed. This discrepancy was because many samples identified as heterozygotes by RFLP-PCR showed very high signal of one dye but very low signal of the other dye in qPCR. Thus, these samples did not show the expected fluorescence. For example, most of the samples for *Taq*I SNP showed very high fluorescence of FAM but almost no signal for VIC. As a result, no sample was found

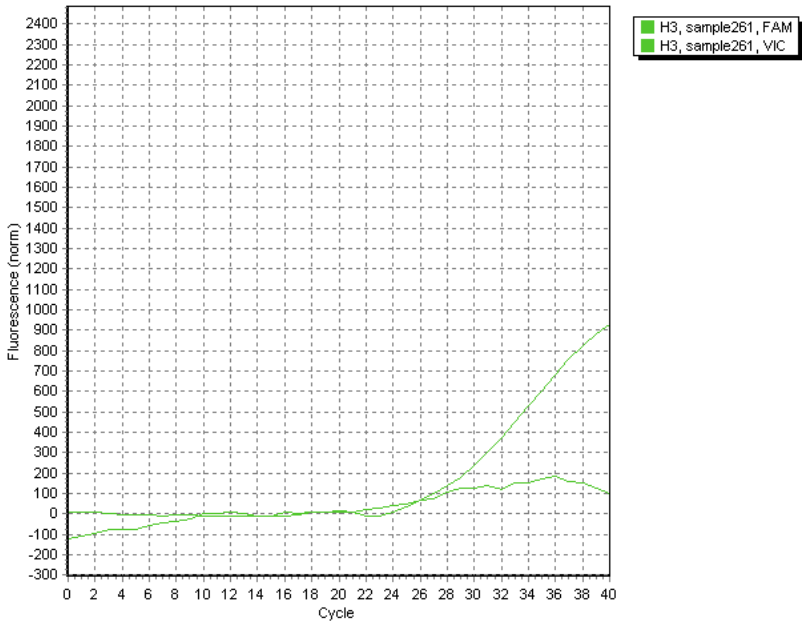
to be homozygote for VIC which detected the T allele (Table 3.1). Table 3.1 compares the frequencies of genotypes for all three SNPs obtained from qPCR and RFLP-PCR. The signal of VIC was so low with Applied Biosystems MicroAmp^{TM} Optical 96-well reaction plates that it was barely positive for samples which were heterozygotes. Therefore, the Applied Biosystems MicroAmp^{TM} Optical 96-well reaction plates were replaced by Eppendorf twin.tec real-time PCR plates to increase the sensitivity to detect VIC signal which was much lower in the Applied Biosystems plates. As a result, a slight increase in the detection of the VIC signal was observed when Eppendorf twin.tec real-time PCR plates were used. Even though the two assays shown in Figures 3.3A and B are different, it is evident that sample 261 which is a heterozygote for *Taq*I and *Bsm*I SNPs show fluorescence for VIC dye in *Bsm*I assay when Eppendorf plates were used but not in *Taq*I assay when Applied Biosystems plates were used. This increase of the VIC signal was evident by much lower Ct (threshold cycle) values of the VIC of many samples which meant that the efficiency of the PCR reaction had increased and more molecules of DNA were amplified. However, few positive controls and samples still did not show the expected fluorescence.

Table 3.1. Comparison of genotypic distributions of the three SNPs by qPCR and RFLP-PCR with their percentages among 239 South-Asian women

		Frequency (%)	
SNPs	*Genotypes*	*qPCR*	*RFLP-PCR*
BsmI	BB	1 (0.4%)	46 (19.3%)
	Bb	194 (81.5%)	119 (49.8%)
	Bb	43 (18.1%)	74 (30.9%)
Total		238*	239
ApaI	AA	75 (31.4%)	77 (32.2%)
	Aa	100 (41.8%)	110 (46%)
	Aa	46 (19.2%)	52 (21.8%)
Total		221*	239
TaqI	TT	0	113 (47.3%)
	Tt	156 (65.3%)	103 (43.1%)
	Tt	69 (28.9%)	23 (9.6%)
Total		225*	239

*- the total number of individuals differ from 239 as some samples showed very low or no fluorescence for one of the dyes.

(A)



(B)

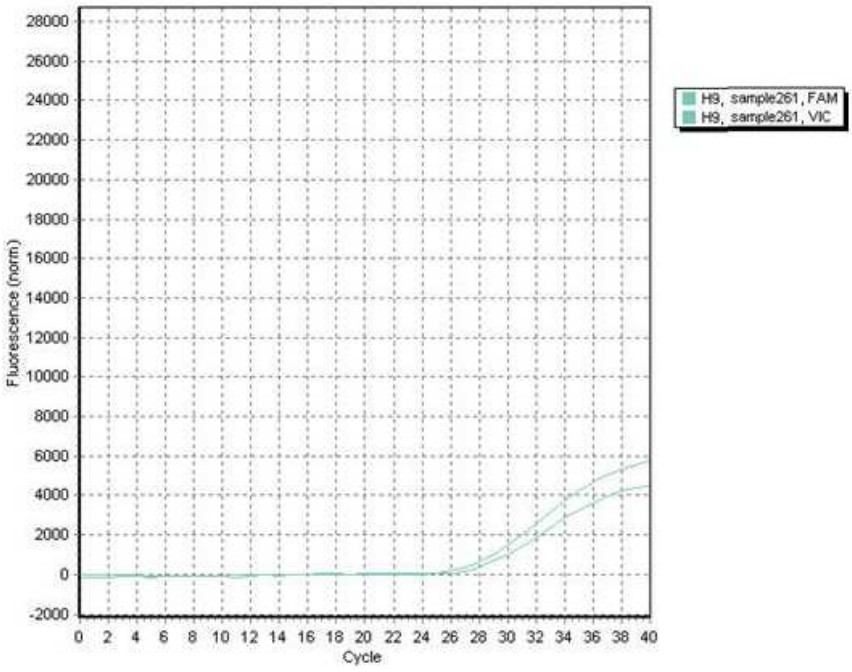
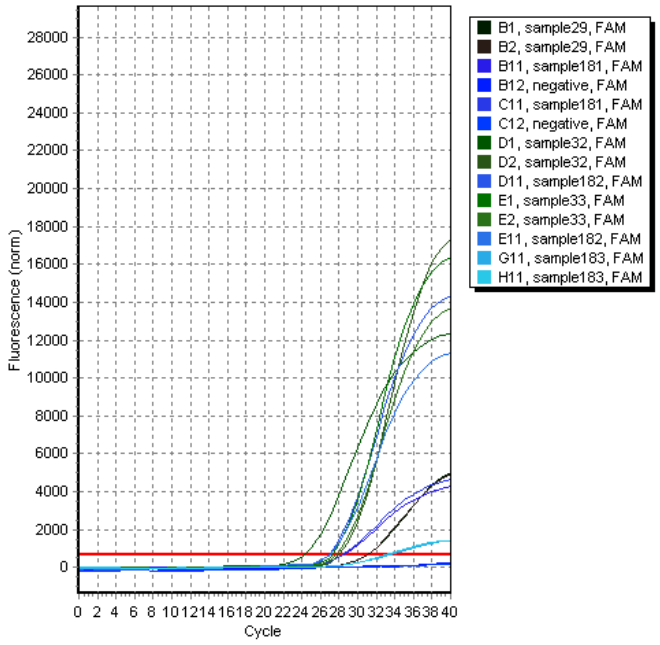


Figure 3.3. Detection of fluorescence of dyes of sample 261 by qPCR for (A) *Taq*I and (B) *Bsm*I assays using Applied Biosystems MicroAmp^{TM} Optical 96-well reaction plates and Eppendorf twin.tec real-time PCR plates, respectively.

The positive homozygous and heterozygous controls used in qPCR for all three SNPs had been identified previously by RFLP-PCR and were confirmed by sequencing. However, it was observed that the positive homozygous controls for each SNP did not show the expected fluorescence for their respective alleles/dyes (either VIC or FAM). Rather they behaved as heterozygotes showing fluorescence for both VIC and FAM. Thus, the threshold could not be set according to the positive controls which resulted in inaccurate identification of zygosity of other samples as the samples did not show the expected fluorescence for either VIC or FAM. Figures 3.4 A and B show the *Bsm*I assay on few samples using the Eppendorf twin.tec real-time PCR plates. The positive heterozygote for *Bsm*I assay was sample 181 and positive homozygote for FAM (allele B) and VIC (allele b) were samples 182 and 183, respectively. So, when the threshold was tried to set according to the samples 181 and 182, sample 183 (homozygote control for VIC) also showed fluorescence for FAM (Figure 3.4 A). Thus, the threshold could never be set according to all three positive controls. Similarly, the threshold could not be set according to the positive controls for VIC as in this case, sample 182 behaved as heterozygote showing fluorescence for VIC (Figure 3.4 B). As a result, samples 29, 32 and 33 were found to be heterozygotes for *Bsm*I SNP, that is, they showed fluorescence for both the dyes. However, samples 29 and 33 were identified as homozygotes by RFLP-PCR for VIC and FAM, respectively. This showed that since the threshold could not be set according to the three positive controls, all the other samples showed unexpected fluorescence. One could sequence few samples to confirm the reliability of the technique. However, for this study only the positive controls for the qPCR were sequenced and their zygosity matched with the results of RFLP-PCR. Thus, results of RFLP-PCR were more reliable than qPCR.

(A)



(B)

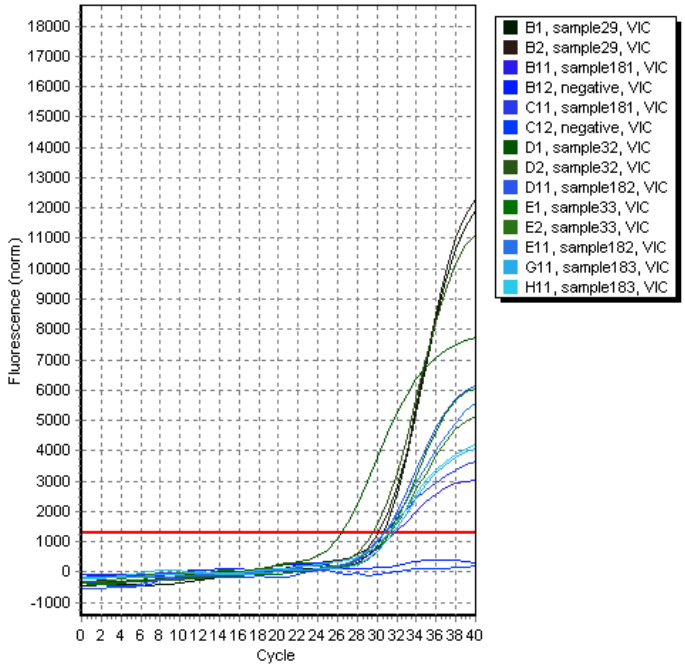
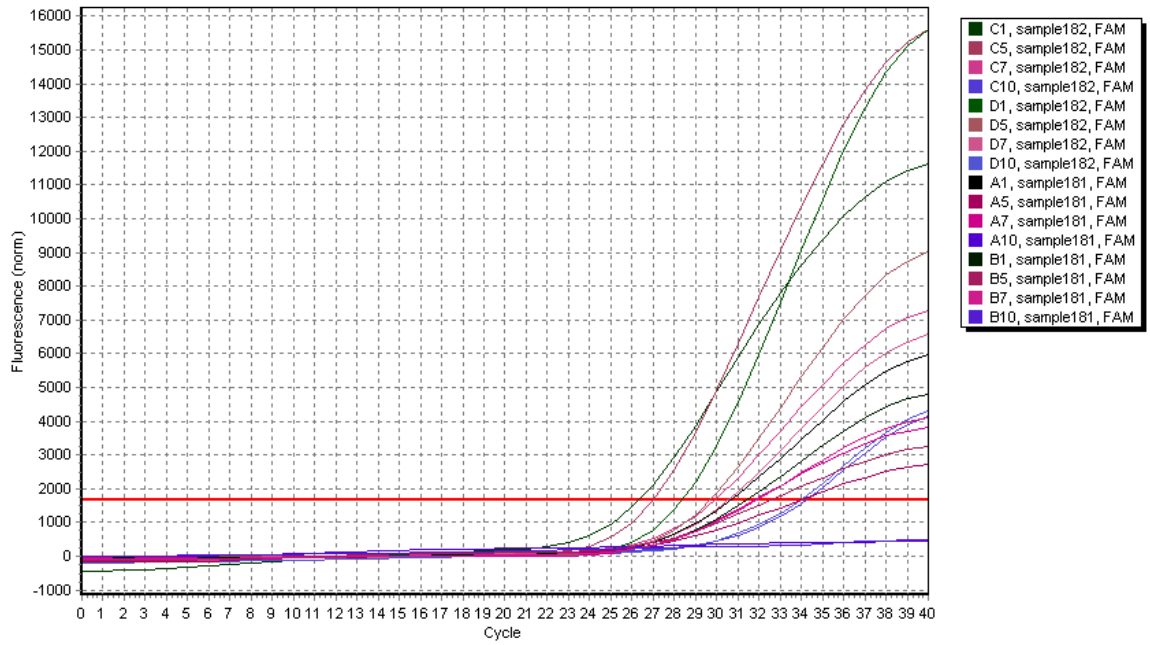


Figure 3.4. Amplification plots for VIC and FAM showing threshold (red- line) for *Bsm*I. The threshold was set according to positive controls. Fluorescence for FAM and VIC indicated positive for B and b alleles, respectively. Figure 3.4A shows samples 29, 32 and 33 that were positive for FAM and figure 3.4B shows the same samples positive for VIC. However, samples 29 and 33 were homozygotes for VIC and FAM, respectively and so the threshold could not be set at the correct level giving rise to false positives.

Various attempts were made to eliminate the false positives or negatives. The first attempt was to re-extract the gDNA of the homozygote positive samples to eliminate the possibility of contamination but they yielded the same results in the qPCR, behaving as heterozygotes. Secondly, a gradient PCR at 60^{o}C, 62^{o}C, 64^{o}C and 66^{o}C was undertaken to determine the precise annealing temperature at which both the probes bind to their respective alleles giving distinct fluorescence at a particular temperature (Figure 3.5). However, all the attempts to eliminate false positives and negatives failed as the homozygote positive controls behaved as heterozygotes. Comparison of the genotype frequencies as determined by qPCR with that determined by RFLP-PCR showed a large disparity between the data. The error in the allele calls using the qPCR was found to be unacceptably high.

(A)



(B)

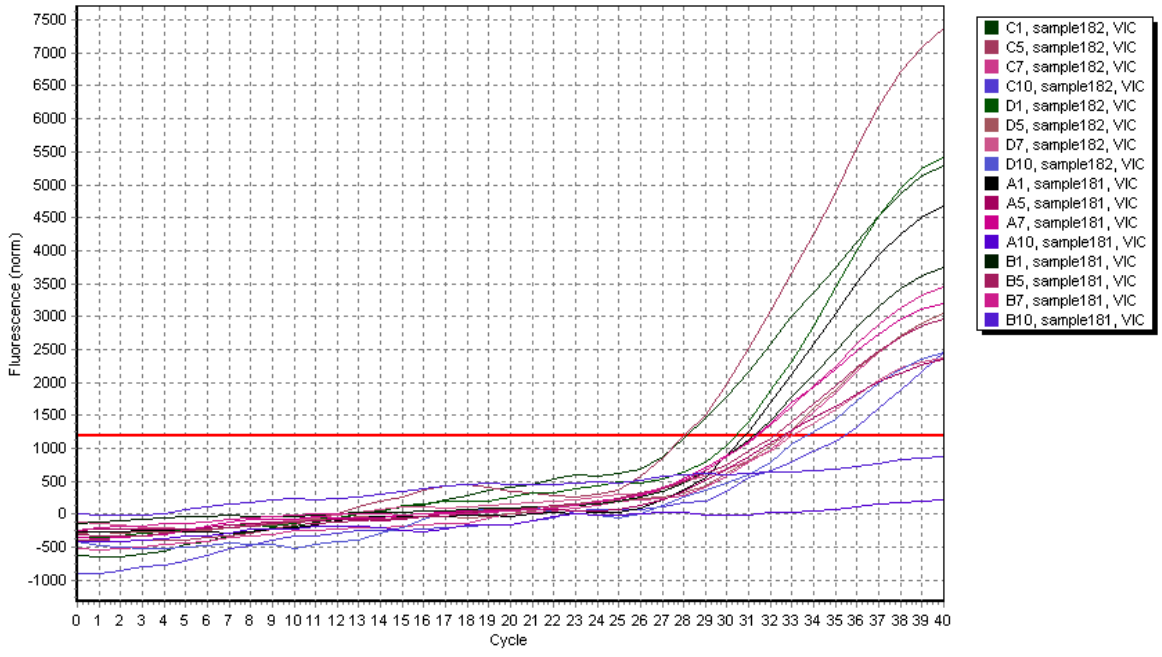


Figure 3.5. Gradient PCR performed to determine the precise annealing temperature of the probes labelled with (A) FAM and (B) VIC for *Bsm*I assay. The positive homozygote controls (samples 182 and 183) for *Bsm*I SNP behaved as heterozygotes. No fluorescence was observed for sample 181 at 66°C. The temperature of A^1, B^1, C^1 and D^1 was 60°C, A^5, B^5, C^5, D^5 at 62°C, A^7, B^7, C^7, D^7 at 64°C and A^{10}, B^{10}, C^{10}, D^{10} at 66°C.

3.4. Discussion

The qPCR technique was compared to RFLP-PCR as a technique for detecting genotypes of *VDR* SNPs *Bsm*I, *Apa*I and *Taq*I. The results presented here indicate RFLP-PCR is a more reliable method than qPCR for genotyping loci at the 3' end of the *VDR* gene. There are many associated pros and cons for each technique. As mentioned earlier, qPCR resulted in heterozygote calls for known homozygotes as the latter showed fluorescence for both the dyes. A possible explanation, especially in the case of *Taq*I locus, could lie in the signal intensity of VIC allele. To overcome this, Applied Biosystems MicroAmp^{TM} Optical 96-well reaction plates were replaced by Eppendorf twin.tec real-time PCR plates which resulted in a slight increase in the detection of the VIC signal. This increase in signal was because of the white wells of the Eppendorf plates that reflect fluorescence of low volume PCR reaction mixture much better than clear wells of Applied Biosystems plates. The low volume PCR reaction mixtures often have very low fluorescence intensity due to small volumes of the components of the PCR and may remain undetected with the clear wells. Thus, the low intensity of the VIC signal slightly increased by using the right consumables (Eppendorf twin.tec real-time PCR plates) with the same instrument (Eppendorf Mastercycler EP Realplex machine). Another drawback was that in qPCR, the positive homozygotes showed fluorescence for both the dyes, VIC and FAM, that is. they behaved as heterozygotes. A possible explanation is that since the probes (VIC and FAM) for the two alleles of a SNP (for example *Taq*I) differ only by one nucleotide (A/G), there may have been cross-binding of probes, resulting in the signal of the other dye. Cross-binding occurs when one of the probes hybridises to the complementary target sequence of the other probe. Various attempts were made to eliminate cross-binding. After the first attempt of re-extraction of DNA from buffy coats of positive controls, the continued appearance of unexpected signals of these positive samples only showed that there was no DNA contamination of these samples. The second attempt of a gradient PCR was undertaken to determine the precise annealing temperature at which each probe binds to the cognate allele, thus preventing the crossbinding of probes and resulting in a clear distinction of the fluorescence of the two dyes. However, all attempts to eliminate crossbinding failed and thus it was concluded that the design of the primers/probes are not adequate for allelic

discrimination using the conditions as prescribed by Applied Biosystems for their 'off the shelf' SNP assay kits.

Another drawback associated with qPCR is that it requires a degree of expertise and technical know-how regarding setting the threshold levels. The setting of threshold levels relies on accurate allele identification within the positive controls. The threshold levels are established by the operator as the minimum levels of fluorescence required for a sample to be considered positive. This level is judged by the fluorescence intensity of positive and negative controls. In this study, the homozygous positive controls were largely positive for both dyes, so it was impossible to set the threshold with any accuracy. This resulted in incorrect reading of fluorescence of other samples which affected their zygosity.

In addition to the above, the genotypes for some of the samples for *Bsm*I (n=1), *Apa*I (n=18) and *Taq*I (n=14) could not be determined by qPCR because the concentration of DNA was too low; the signal intensity after 40 cycles of amplification failed to cross the FAM or VIC threshold settings. Applied Biosystems suggest the use of only 2 µl of buffy coat sample to yield enough DNA to be detected by qPCR. The DNA concentration was not measured, so the exact amount of template was unknown. It could be that because the starting concentration was so low, it was not robust enough for amplification by qPCR.

Even though RFLP-PCR is a more reliable technique than qPCR, the former technique produces non-specific fragments which were either produced in PCR or were a result of incomplete digestion. Therefore, individual digestions by each enzyme had to be undertaken in order to differentiate between the digested and partially digested fragments. It takes more time to set-up RFLP-PCR, but it is much cheaper and apparently more reliable than qPCR. qPCR, when coupled with properly designed probes, could be a very useful technique for a very rapid determination of the frequencies of any SNP. Also, the DNA extraction procedure is faster and the assay requires less DNA. Applied Biosystems claims that these custom-based assays are tested but it appears that the assays described here failed to determine genotypes in a reliable manner. Also, unlike RFLP-PCR, it cannot be used to determine the haplotype of an individual as only one SNP is assayed at a time.

Based on the observations made while comparing the two techniques, the hypothesis stated earlier that qPCR is much more reliable than RFLP-PCR was not supported. For *VDR* SNPs, these primers and probes do not seem to be tested by Applied Biosystems for successful discrimination of alleles and the assay failed completely.

Chapter 4

Analysis of association between the *VDR* gene SNPs and insulin resistance/sensitivity

4.1. Introduction

Vitamin D deficiency has been shown to play a role in several clinical conditions (see chapter 1). Since vitamin D exerts its effects through the VDR, nucleotide changes in the *VDR* gene may affect transcript levels, transcript stability or the functional integrity of the VDR protein in such a way that downstream vitamin D pathways are adversely affected. Most of these nucleotide changes in the *VDR* gene occur as SNPs which have been associated with diseases such as osteoporosis, cancer and diabetes.

4.1.1. Bone related studies

Osteoporosis is a 'diminution of skeletal mass in which bone is normally mineralised, but the amount of bone tissue in a given volume of bone is reduced, causing mechanical weakness and leading to fractures especially of the hip and spine, which can occur either spontaneously or with minimal trauma. One of the primary predictors of this fracture risk is Bone Mineral Density (BMD) (g/cm^2)' (Thakkinstian, D'Este, Eisman, Nguyen & Attia, 2004, p.419). Recent studies have suggested that the *Bsm*I BB genotype is associated with low BMD at the hip compared with the bb genotype (presence of *Bsm*I site) (Fang et al., 2006). The association of a *Bsm*I allele with BMD is significant, but results are conflicting as to which allele (B or b) is the risk allele associated with low BMD (Thakkinstian et al. 2004; Uitterlinden et al., 2002). Uitterlinden et al. (2006) reported that *Fok*I, *Bsm*I, *Apa*I and *Taq*I polymorphisms are not associated with BMD or with fractures but there is a possible association of Cdx-2 SNP with risk of vertebral fractures. It is not surprising that the Cdx-2 locus was found associated with a risk of vertebral fractures as the A allele has been shown to increase production of calcium transport proteins by increasing transcription of the *VDR* gene that in turn affect calcium uptake. This influences BMD and susceptibility to fractures in the elderly (Fang et al., 2003; Jehan et al., 2007; Lins et al., 2007; Uitterlinden et al., 2004). It could be predicted that an individual homozygous for the A allele (AA) would have the highest rate of the *VDR* gene transcription and therefore highest calcium absorption. Therefore, individuals with an AA genotype may have a lower risk of fracture as they would take up calcium at a greater rate (Fang et al., 2003; Uitterlinden et

al., 2006). Other studies that have focussed on associations between bone health and SNPs in the *VDR* gene, found no association between the Cdx-2 SNP and bone health or the risk of fracture (Barr, Stewart, Torgerson, & Reid, 2010; Macdonald et al., 2006). It is recognised that these genetic studies differ in their results due to the ethnicity of the populations under study (Macdonald et al. 2006). The frequency of an allele and thereby genotypes differ from population to population due to racial differences, environmental background and evolutionary history (Macdonald et al. 2006). For example, the A allele is more prevalent in African subjects than their white counterparts (74% versus 19%) which may explain higher BMD in the former population in spite of low calcium intake (Macdonald et al., 2006). The second reason could be that diseases related to bone health are complex and multifactorial such that they are influenced by gene-gene and gene-environment factors and so any association between bone health and *VDR* SNPs may be weak and so below the level of detection (Macdonald et al., 2006). Thirdly, the sample size has varied from study to study resulting in some studies having limited statistical power. Fourthly, the varying strength of LD between certain haplotypes and a potential neighbouring bone-metabolism related gene may exist in different ethnic groups which could give rise to recombination between the gene and particular haplotypes in some populations but not in others. Finally, there may be allelic heterogeneity i.e. the *VDR* gene may be associated with osteoporosis, but different gene variants may be implicated with osteoporosis in different populations (Uitterlinden et al., 1996). For example, a recent study showed that participants with the genotypes *Fok*I FF along with *Apa*I aa, *Bsm*I bb and *Taq*I TT had 10% higher BMD in the spine and hip compared to those with the genotypes ff, AA, BB and tt, respectively (Mitra et al., 2006).

The ATG start codon of the VDR protein is polymorphic in that the 'T' position may be a 'C' residue. In the event of the C allele, translation begins 9 bp downstream at the next ATG codon. The presence of the T allele creates a *Fok*I (f allele) restriction enzyme site whereas a 'C' abolishes the site (F allele) and the protein of 424 amino acids (M4 form) which is shorter by three amino acids (Uitterlinden et al., 2004). The shorter form of the VDR protein is more active than the longer form (M1 form) (Arai et al., 1997; Jurutka et al., 2000; Uitterlinden et al., 2004). Research on *Fok*I genotypes has mostly associated them with cancers or fracture susceptibility as this SNP affects the protein sequence.

Studies have suggested that the *Fok*I f allele is associated with decreased calcium absorption and thus with low BMD (Davis, 2008). Therefore, individuals with ff genotype would be expected to have increased risk of fractures (Mitra et al., 2006).

Although numerous molecular association studies indicate that *VDR* genotypes are associated with BMD, there are few studies that have examined the association of haplotypes with BMD. Meta-analyses have associated haplotypes with body height, body weight and risk to osteoporotic fracture in Caucasians (Fang et al., 2005, Fang et al., 2007). Thakkinstian et al. (2004) indicated that osteoporosis was significantly associated with haplotypes BAt and bAT in Caucasians but not with individual polymorphisms. This conclusion was partly supported by the study of Uitterlinden et al. (1996) that reported an association between a decrease of BMD at the femoral neck by 0.014 g/cm^2 per copy of bAT in Caucasians. An association of the most frequently occurring haplotypes baT and/or BAt with diseases could be due to linkage of the former with a large number of 'A's (*n*=18-24) in a polyA VNTR at the 3' end, while the latter is linked to a smaller number of 'A's (*n*=13-17). The number of 'A's could influence the stability of VDR mRNA thus affecting its half-life (Morrison et al., 1994).

4.1.2. Cancer related studies

Many studies have examined the role of SNPs in relation to cancer risk. Research has shown that calcium can help in chemoprevention of colon cancer (Kállay, Bonner, Wrba, Thakker, & Peterlik, 2002). Therefore, it may be expected that individuals homozygous for Cdx-2 AA genotype, which is associated with increased calcium absorption from the intestine, would be protective against colon cancer compared to GG and AG genotypes. In addition, individuals carrying the *Fok*I ff genotype which is associated with decreased calcium absorption, may be more susceptible to colon cancer compared to FF and Ff genotypes. However, Ochs-Balcom et al. (2008) found that Cdx-2 A in addition to *Fok*I f alleles are associated with an increased risk of colon cancer in low adiposed Caucasians although another study reported no association of *Fok*I alleles with colon cancer (Slattery, Yakumo, Hoffman, & Neuhausen, 2001).

*Fok*I SNP genotypes have been linked to various other forms of cancer, but the studies have been inconsistent. For example, an association of the ff genotype with more aggressive prostate tumours in the presence of low plasma 25(OH)D levels has been demonstrated (Li et al., 2007). However, some studies did not show any association between prostate cancer and *Fok*I alleles (Bai et al., 2009). Mittal, Manchanda, Bhat, and Bid (2007) reported a two-fold increased risk in bladder cancer among people carrying the FF genotype. Meta-analyses by Ntais, Polycarpou, and Ioannidis (2003) and Berndt, Dodson, Huang, and Nicodemus (2006) reported on 14 and 16 studies, respectively, and found no association of the *Fok*I, *Taq*I and *Bsm*I SNPs with prostate cancer.

An association of reduced breast cancer risk with the *Bsm*I B allele has been reported (McCullough et al., 2009). However, another study reported the bb genotype to be associated with reduced breast cancer risk (Ingle et al., 2001). These epidemiological studies have repeatedly reported that the ethnicity of the population influences the association of *VDR* SNPs with cancer risk (Slattery, 2007). Significant associations have been demonstrated between the diet (calcium and vitamin D intake) and cancer, providing an alternative reason for observed associations (Kim et al., 2001). Therefore, it is important that while analysing results, confounding factors such as diet should be considered to give more accurate results.

4.1.3. Other studies

Apart from studies focussing on cancer risk and bone related health problems, *Apa*I and baT have been associated with age-related changes in better cognitive functioning and in less depressive symptoms in contrast to *Bsm*I, *Taq*I and BAt in 85 year old inhabitants of The Netherlands (Kuningas et al., 2009). Vitamin D has been shown to play a role in determining the risk for multiple sclerosis (MS). Therefore, Dickinson et al. (2009) analysed *VDR* gene SNPs, which are critical in the vitamin D pathway and found an association between the Cdx-2 polymorphism, low sun exposure and MS. They reported an association between the ‘G’ allele within the Cdx-2 SNP and increased risk of MS with low sun exposure in early childhood.

4.1.4. Introduction to insulin resistance

Insulin is a peptide hormone produced by β cells of the pancreas that regulates the metabolic functions of the body and is a primary cause for diabetic syndromes (Taylor et al., 1991). Defects in insulin production or secretion can lead to cardiovascular diseases, hypertension, dyslipidemia and other metabolic syndromes (Ferrannini, 2006). Insulin resistance is the lack of responsiveness of cells and tissues to insulin levels, resulting in the dysregulation of glucose homeostasis. This may give rise to increased levels of insulin synthesis and secretion by the pancreas (Muniyappa, Montagnani, Koh, & Quon, 2007).

Since vitamin D is a fat-soluble vitamin, it is deposited in the subcutaneous fat in obese people, thus becoming bio-unavailable. These individuals may suffer from insulin resistance due to the lack of vitamin D. While weight loss would benefit these individuals who are insulin resistant and increase their sensitivity to insulin, not all obese individuals are insulin resistant and those who are, would not necessarily return to normal levels seen in insulin-sensitive individuals (Reaven, 2005). Similarly, not all normal-weight individuals are insulin sensitive, nor are they spared from developing symptoms of insulin resistance (Reaven, 2005). Since there is no simple method to measure insulin resistance in individuals, either all obese individuals must lose weight irrespective of their insulin status or efforts should be initiated to find alternatives that will help health care professionals to identify individuals who are insulin resistant. Health care professionals usually rely on the latter option and have various formulae that measure fasting serum glucose/ insulin. These formulae help them to identify insulin resistant individuals (Reaven, 2005).

4.1.4.1. Homeostasis model assessment

One of the measures that is widely used to estimate insulin resistance is the Homeostasis model assessment (HOMA). This approach was developed in 1985 and relies on the measurement of fasting serum glucose (FSG) and fasting serum insulin (FSI) in a linear equation to measure insulin resistance (HOMA-IR) (Bikle, 2009a, 2009b). Insulin sensitivity (HOMA%S) is the reciprocal of insulin resistance. This computer model was later amended to improve its predictability of insulin resistance to better reflect human

physiology. The revised non-linear model called HOMA2 allows the determination of insulin sensitivity (HOMA2%S) from FSG and FSI (Wallace, Levy, & Matthews, 2004). HOMA2%S is expressed as a percentage where 100% is considered normal. HOMA2-IR is the reciprocal of insulin sensitivity (HOMA2%S) and a value of 1 is considered normal and values greater than 1 indicate high insulin resistance (Levy, Matthews, & Hermans, 1998).

4.1.4.2. Association studies

The association of insulin resistance with vitamin D levels and how vitamin D can improve insulin sensitivity have already been described in chapter 1. Genetic association studies have reported a link between *VDR* gene polymorphisms and insulin sensitivity/resistivity, type 2 diabetes and obesity. Certain polymorphisms in the *VDR* gene can change the protein structure, or regulate levels of VDR mRNA and its protein levels, thereby affecting the downstream proteins (Ogunkolade et al., 2002). Despite little data and conflicting results, such genetic research is crucial in understanding the role of vitamin D and VDR in the commencement of such diseases (Song & Manson, 2010). A study by Ogunkolade et al. (2002) reported an association of the *Taq*I tt genotype with higher levels of insulin secretion in a cohort of 143 Bangladeshi adults. A subset of these individuals (n=93) were vitamin D insufficient with serum 25(OH)D <20 ng/ml. In these individuals *Fok*I in addition to *Taq*I were found to be significant determinants of insulin secretion. However, the genotypes of *Fok*I and *Taq*I SNPs that were associated with insulin secretion in these vitamin D insufficient individuals were not mentioned. They justified their observation by stating that *Fok*I and *Taq*I SNPs were in LD and so it is a possibility that both were associated with the same phenotype. However, no studies have reported LD between *Fok*I and *Taq*I except this study. They also showed that *Fok*I FF and *Taq*I TT genotypes were associated with higher levels of VDR mRNA compared to the tt and ff genotypes and *Taq*I TT was also associated with highest VDR protein levels. Most studies have found an association between insulin sensitivity/resistivity with either *Bsm*I or *Fok*I variants. For example, Chiu et al. (2001) reported an association of *Fok*I Ff and ff genotypes with lower insulin sensitivity (HOMA%S) than FF genotype in healthy Caucasians but no effect on β cell function or insulin secretion. Other examples of studies are given in Table 4.1.

Table 4.1. Association between *VDR* gene variants and phenotypes related to glucose intolerance

Population origin/ethnicity	*At risk allele/ genotype*	*Phenotype observed*	*Reference*
Non-diabetic Caucasians	Bb	High insulin resistance (HOMA-IR)	Oh, & Barrett-Connor, 2002
	Aa	Glucose intolerance	
Randomly selected men in Poland	FF and Ff	Insulin sensitivity	Filus et al., 2008
Patients with Type 2 diabetes mellitus in Tehran	Ff and ff	Higher HOMA-IR	Hossein-nezhad et al., 2009
Postmenopausal women in Poland	No association of *Bsm*I SNP	Insulin resistance	Tworowska-Bardzinska et al., 2008

4.1.2.3. The present study

In 2006, the New Zealand Ministry of Health published the first comprehensive report of the Asian and South-Asian community focussing on specific health issues of particular importance. According to this report, hospitalisation and mortality rates due to cardiovascular diseases (CVD) for all age groups and both sexes were significantly higher in Indian people compared to the Asian population. The significantly higher incidence of CVD in the Indian population could partly be explained by the high incidence of type 2 diabetes in Indian people. This was as high as 9.4 (3.9-15) self reported cases per 100 in Indian peoples (according to 2002/2003 New Zealand Health Survey) compared to 3.4 (0.6-6.3) and 5.7 (1.8–9.6) cases per 100 in Chinese and other Asian groups, respectively. Thus, it can be inferred that incidence of CVD and type 2 diabetes is significantly higher in the Indian population than the rest of the Asian populations (Ministry of Health, 2006). The population of interest in this study is South-Asian women living in Auckland who have low vitamin D levels (25(OH)D <80 nmol/l). A high incidence of hypovitaminosis D is found in this cohort in addition to symptoms of insulin resistance leading to type 2 diabetes. Very

little is known about the genetic predisposition of this population to insulin resistance, and there are few studies that have reported an association of *VDR* polymorphisms with both type-1 and type 2 diabetes in the Indian population (Bid et al., 2009; McDermott et al., 1997).

Since studies have shown an association between *VDR* gene polymorphisms and insulin resistance in many ethnic groups, it was considered worthwhile to investigate if there were an association between *VDR* gene SNPs and insulin resistance and the change in insulin resistance to vitamin D supplements, that is, how each individual possessing different genotypes responds to vitamin D supplements. Each SNP has two allelic variants that combine to form three genotypes. For example, *Fok*I SNP has F and f variants and they combine to give three genotypes, FF, Ff and ff. The combination of alleles of various SNPs along the length of a chromosome gives the haplotype. For this study, the genotypes of five SNPs and haplotypes at three clustered anonymous restriction enzyme sites were determined and their association with insulin resistance was investigated.

There were two hypotheses for this study. First that there is an association between the *VDR* gene SNPs, Cdx-2, *Fok*I, *Bsm*I, *Apa*I and *Taq*I with insulin resistance/sensitivity in 239 South-Asian women who have serum 25(OH)D<80 nmol/L (Phase1). Secondly, individuals from this cohort who have serum 25(OH)D<50 nmol/L will respond differently to vitamin D supplementation according to the SNPs they have. The response assessed here would be a change in insulin sensitivity.

The aims of this project were two fold. The first aim was to investigate the association between insulin resistance and *VDR* SNPs (Cdx-2, *Fok*I, *Bsm*I, *Apa*I and *Taq*I) in 239 South-Asian women (Phase 1)**.** The second aim was to investigate the effect of *VDR* genotypes on the response to insulin sensitivity in vitamin D supplemented group as compared to the placebo group (Phase2).

4.2. Methods and Materials

4.2.1. Participants

Genetic analysis was carried out on 239 South-Asian women living in Auckland who participated in the Surya Study of Hurst et al. (2008). The Surya Study consisted of two phases: Phase 1 and phase 2. Phase 1 included initial recruitment, general screening of 239 subjects and biochemical data was collected. Phase two consisted of eighty-one individuals who were selected on the basis of certain criteria and one of them was high insulin resistance (HOMA-IR>1.93) and low vitamin D levels (25(OH)D<50 nmol/L). These individuals participated in a randomised placebo-controlled trial to investigate the effect of improved vitamin D status on insulin sensitivity. HOMA-IR and HOMA2%S were calculated as the indices of insulin resistance and sensitivity, respectively.

4.2.1.1. Phase 1 of the Surya Study

Participants were 239 South-Asian women, living in Auckland, New Zealand and aged 20 years and over. These women were non-diabetic, serum 25(OH)D<80 nmol/L and were either born on the Indian subcontinent or both parents/one of the grandparents was born there. Volunteers having vitamin D supplements of more than 1000 IU/d or calcitriol were excluded from the study and those who participated did not have any severe impairment or disease (Hurst et al., 2008).

4.2.1.2. Phase 2 of the Surya Study

Randomised controlled double-blind intervention was carried out for six months where forty-two subjects were given 4000 IU/d vitamin D_3 and thirty-nine were given a placebo. These subjects were selected on the basis of high insulin resistance (HOMA-IR>1.93) or hyperinsulaemia (FSI>13 mIU/L) or hyperglycaemia (FSG 5.6-7.2 mmol/L) or a triglyceride/High Density Lipoprotein-Cholesterol ratio>3 or serum 25(OH)D<50 nmol/L. There is no consensus regarding the cut off value for HOMA-IR in this population and so

HOMA-IR >1.93 was based on the upper quartile as determined in Chennai Urban Population Study-7 [CUPS-7] (Deepa, Shanthirani, Premalatha, Sastry, & Mohan, 2001). The subjects were matched into pairs by age and body mass index (BMI) and then randomly assigned to receive 4000 IU of vitamin D_3 (n=42) or a placebo (n=39) for six months. Analyses were carried out while these factors were already adjusted. For more details, refer to the study by Hurst et al. (2008).

4.2.2. Funding and ethics

Refer to the appendix, page 166.

4.2.3. Biochemical measurements

Biochemical measurements of these 239 women were carried out as part of the Surya Study and details have been mentioned by Hurst et al. (2008). Those of relevance here were BMI, HOMA-IR and HOMA2%S. The latter two are well known indices for measuring insulin resistance and insulin sensitivity, respectively. HOMA-IR was used for assessing insulin resistance in 239 women in phase 1, and HOMA2%S was used for phase 2 participants. These indices use FSG and FSI and were already calculated as part of the Surya Study.

4.2.4. Genotyping

Blood samples were taken from each participant and a buffy coat removed and stored at -80^{o}C. gDNA was extracted and genotyping for *VDR* SNPs by RFLP-PCR and ASM-PCR was carried out as described in section 2.2.

4.2.5. Statistical measurements

Statistical analyses were performed using the SPSS package for windows version 17 (SPSS Inc., Chicago, IL, USA). Genotype frequencies were examined for compliance with Hardy Weinberg equilibrium using χ^2 analysis for the whole group (239 subjects) and for 81 subjects who entered intervention. Box plots were used to represent the analyses between the genotypes/ haplotypes and insulin resistance for phase 1 and phase 2.

4.2.5.1. Statistics for Phase 1

The baseline characteristics of the 239 women in phase 1 are presented in Table 4.2. Normally distributed data were represented as mean ± standard deviation (SD) and data not normally distributed as median (25^{th}, 75^{th} percentile). The insulin resistance was measured by HOMA-IR scores and its association with genotypes/haplotypes was assessed by multiple regression analyses. In Table 4.4, the data for HOMA-IR were not normally distributed and so were log transformed to achieve a normal distribution, and the data were presented as geometric mean with 95% confidence interval (95% CI). Normality was tested using Kolmogorov-Smirnov and Shapiro-Wilk tests and homogeneity of variance between groups was tested using Levene's test. Multiple regression analysis was used to examine an association between *VDR* genotypes/haplotypes and insulin resistance measured by HOMA-IR while adjusting for BMI since the latter is a significant predictor of insulin sensitivity/resistance. Since multiple regression analysis cannot be used for variables with more than two categories, dummy variables were used to represent categories (genotypes) with ones and zeros. In the case of multiple regression analysis with genotypes, homozygous groups were selected as controls. The particular homozygous group used as a control for each SNP was that which had the higher frequency, that is, more commonly occurring in 239 participants. For example, *Bsm*I bb genotype (n=74) was selected as the control group as compared to BB genotype (n=46). The homozygous group was selected as the control group as a relationship could be observed between two extreme groups (BB vs bb) than between heterozygous and homozygous groups (Bb vs bb).

Allele dose was defined as the number of copies of a particular allele. For example, an individual with haplotype genotype 'baT-baT' has two copies of haplotype allele 'baT'. Allele dose effect on insulin resistance (HOMA-IR) was determined using multiple regression using no copy for that particular haplotype allele as control group. For example, in case of baT, no copy of baT was selected as a control group to compare it against one and two copies of baT. These allele dose effect analyses were done for only two haplotype alleles which had the highest frequencies (Table 2.5) as the frequencies for the rest of them were very low for analysis.

An association between insulin resistance (HOMA-IR) and three most frequent haplotype genotypes were also analysed using multiple regression. The remainder had too low frequencies for analysis (Table 2.5). All assumptions for multiple regression analyses were met: residuals were normally distributed and independent (Durbin-Watson test), variance was constant (as assessed by plotting regression standardized residuals against the regression standardized predicted values) and the assumption for multi-colinearity was met (tolerance and VIF values). Age and serum 25(OH)D were not significant predictors for the model, hence they were excluded from the analyses. The results of multiple regression analyses of insulin resistance with genotype, haplotype allele dose effect and haplotype genotype are presented as tables, with boxplots (plotted using SPSS) as the graphical representation of these findings. The level of significance was 0.05.

4.2.5.2. Statistics for Phase 2

Phase 2 consisted of 81 participants of which 42 were given vitamin D supplements and 39 a placebo. Differences in the genotype frequencies between placebo and vitamin D supplemented groups were assessed using χ^2 analysis. The response to vitamin D treatment involved measuring changes in insulin sensitivity that was assessed by HOMA2%S. The change in HOMA2%S over the six-month intervention period was calculated by subtracting the baseline value from the endpoint value. Since the data for HOMA2%S was not normal and could not be transformed (as it is a result of an algorithm), non-parametric tests were used. Kruskal-Wallis test was used to compare the change in HOMA2%S between different

genotypes within placebo and vitamin D groups separately. Post-hoc analysis was done using Mann-Whitney test with Bonferroni adjustments (α/number of comparisons); the level of significance after Bonferroni adjustment was 0.016 (0.05/3). These analyses have been presented as tables, with boxplots (plotted using SPSS) as the graphical representation of these findings.

Analyses with haplotype allele dose and haplotype genotype could not be carried out as the frequencies of the haplotype allele dose and haplotype genotype were extremely small.

4.3. Results

The genotypes for the five SNPs were in Hardy-Weinberg equilibrium for both phase 1 and phase 2 (P>0.05).

4.3.1. Results for Phase 1

Two hundred and thirty nine women were recruited for the Surya Study and their baseline data were collected (Table 4.2).

Table 4.2. Baseline characteristics of 239 participants in the Surya Study (Phase 1)

*Baseline characteristic^*	*Mean± SD*
Age (years)	40.6±10.3
Education (in years)	16.2±3.5
Years in New Zealand	8.8±10.1
BMI (kg/m^2)	25.7±4.3
Serum 25(OH)D	28 (18, 44.3)
Systolic blood pressure (mmHg)	117 (106, 127.3)
Diastolic blood pressure (mmHg)	77.8±10.3
LDL-C (mmol/L)	2.9±0.7
HDL-C (mmol/L)	1.3 (1.1, 1.5)
FSI (mU/L)	9.3 (5.98, 12.9)
FSG (mU/L)	4.7 (4.4, 5)
TG (mmol/L)	1.1 (0.8, 1.6)
HOMA-IR	2 (1.2, 2.8)
TC	4.8±0.8

Abbreviations: BMI-body mass index; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; FSI-fasting serum insulin; FSG- fasting serum glucose; TG-triglyceride; HOMA-IR-Homeostasis model assessment for insulin resistance; TC-total cholesterol.
^Normally distributed data presented as mean ± standard deviation (SD) and data not normally distributed as median (25^{th}, 75^{th} percentile).

The majority of participants were Indians (91%) followed by Sri Lankans (6%) and Pakistanis (3%). These women were above 20 years of age, with an average of approximately 40.6±10.3 years for the whole cohort. They were well educated with the average number of years of education being 16.2±3.5 and had lived in New Zealand for 8.8 ± 10.1 years. Most of the women were not obese although there were few who were. This can be seen from the average of BMI (25.7±4.3). It was observed that they had low average serum 25(OH)D concentrations of 28 (18, 44.3). There is a lot of debate regarding the cut-off values for vitamin D levels to define vitamin D status but the papers published so far consider the above value as low. Both their systolic and diastolic pressures were within the

normal range 120/80 (systolic pressure- 119.2±18.3; diastolic pressure- 77.8±10.3). However, HOMA-IR value was quite high, 2 (1.2, 2.8) compared to the the reference value of 1.93 (Deepa et al., 2001). Considering the reference values for type 2 diabetes risk from Table 4.3, it can be inferred that the average total cholesterol (TC) of this cohort 4.8 ± 0.8 and low density lipoprotein cholesterol (LDL-C) 2.9 ± 0.7, were slightly higher than reference values whereas triglyceride (TG) and high density lipoprotein cholesterol (HDL-C) values were less than expected values. Deviation from the reference values mentioned in Table 4.3 could indicate risk for type 2 diabetes.

Table 4.3. Reference values for optimal risk factor levels for people with diabetes

Lipid fraction	*Value*
TC	<4 mmol/L
HDL-C	>1 mmol/L
LDL-C	<2.5 mmol/L
TG	<1.7 mmol/L

From "Management of type 2 diabetes" (2003). Retrieved 12 May, 2010, from http://www.nzgg.org.nz/guidelines/0036/Diabetes_summary.pdf

The association between insulin resistance (calculated by HOMA-IR) and *VDR* genotypes were first analysed in 239 women (Table 4.4). Cdx-2 and *Fok*I genotypes did not show any association with insulin resistance. *Bsm*I, *Apa*I and *Taq*I genotypes were found to be significantly associated with insulin resistance by multiple regression analyses. *Bsm*I BB (P=0.026), *Apa*I AA (P= 0.035) and *Taq*I tt (P=0.022) genotypes were significantly associated with lower insulin resistance compared to bb, aa and TT genotypes. This was also evident from their low geometric means of insulin resistance {BB=1.7 (1.5-2); AA= 1.8 (1.5-2); tt= 1.5 (1.2-1.9)} compared to bb {2.1 (1.8-2.4)}, aa {2.1 (1.8-2.4)} and TT {2 (1.8-2.2)}. The value of regression coefficients (β) in Table 4.4 can be interpreted as the predicted change in the dependent variable (HOMA-IR) when the value of the independent variable (genotype) changes with one unit. For example, it is predicted that the South-Asian

women with the *Bsm*I BB genotype will have a insulin resistance level of 0.81 (0.61-0.96) fold lower than women with the *Bsm*I bb genotype (P=0.026). The boxplot in Figure 4.1 shows the distribution of insulin resistance levels among 239 women with the *Bsm*I genotypes and that the median of insulin resistance in BB appears to be lower than bb genotype.

Table 4.4. Prediction of insulin resistance (HOMA-IR) by *VDR* genotypes using multiple regression analysis in 239 women in the Surya Study

Genotype (n)	*Regression coefficients (95% CI)(β)^*	*HOMA-IR*[†]	*P*[#]
Cdx-2[*]			
GG (64)[+]	—	2.1 (1.8- 2.4)	—
AG (132)	0.92 (0.79-1.07)	1.9 (1.7- 2.1)	0.267
AA (43)	0.95 (0.78-1.15)	1.9 (1.6-2.33)	0.579
FokI[*]			
FF (139)[+]	—	1.9 (1.7-2.1)	—
Ff (82)	1.00 (0.87-1.15)	1.98 (1.7-2.3)	0.97
ff (18)	0.92 (0.71-1.18)	2.0 (1.5- 2.7)	0.5
BsmI[*]			
bb (74)[+]	—	2.1 (1.8-2.4)	—
Bb (119)	0.91 (0.79-1.06)	2.0 (1.8-2.2)	0.226
BB (46)	0.81 (0.67-0.97)	1.7 (1.5-2.0)	**0.026**
ApaI[*]			
AA (77)[+]	—	1.8 (1.5-2.0)	—
Aa (110)	1.12 (0.97-1.3	2.0 (1.8-2.3)	0.131
aa (52)	1.21 (1.01-1.45)	2.1 (1.8-2.4)	**0.035**
TaqI[*]			
TT (113)[+]	—	2.0 (1.8-2.2)	—
Tt (103)	0.95 (0.83-1.08)	2.0 (1.8-2.3)	0.42
tt (23)	0.77 (0.61-0.96)	1.5 (1.2-1.9)	**0.022**

[†]HOMA-IR was BMI adjusted and was log transformed. Presented are geometric means (95% CI in brackets)
^Presented is the antilog of regression coefficient: the increment or decrement in HOMA-IR as compared to the control group (in brackets is the 95% CI)
Significance of differences versus the control group
[+]Control group
[*]R^2 for models (including genotype and BMI) *Cdx-2*= 0.313; *FokI*= 0.308; *BsmI*= 0.324; *ApaI*= 0.323; *TaqI*= 0.323
Figures in **bold** show $P<0.05$
Prediction equation for *Cdx-2*: log (HOMA-IR)= –1.232+0.076 BMI–0.086AG–0.054AA
FokI: log (HOMA-IR)= –1.292+0.076 BMI+0.003Ff–0.085ff
BsmI: log (HOMA-IR)= –1.217+0.076 BMI –0.09Bb –0.215BB
ApaI: log (HOMA-IR)= –1.386+0.076 BMI+0.115Aa +0.193aa
TaqI: log (HOMA-IR)= –1.243+0.076 BMI–0.056Tt–0.268tt

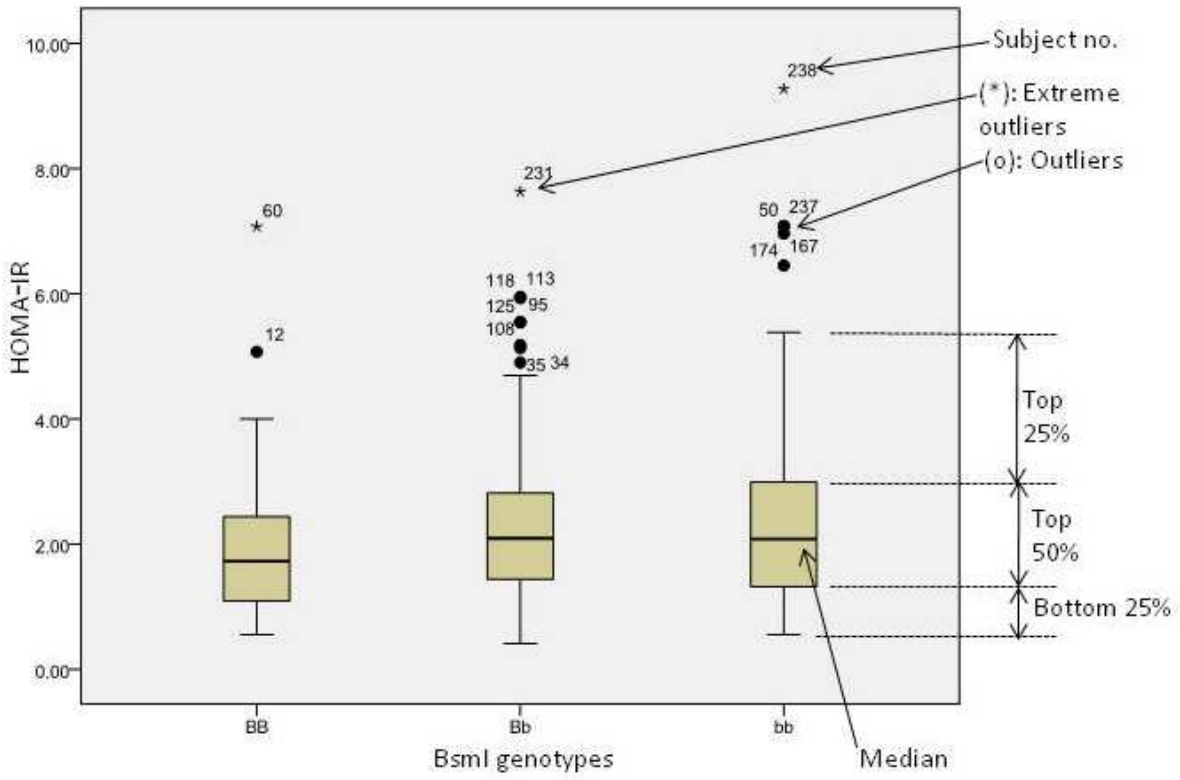


Figure 4.1. A boxplot showing the distribution of insulin resistance (HOMA-IR) scores among *Bsm*I genotypes (BB, Bb and bb) in 239 women.

For *Apa*I, it is predicted that South-Asian women with the *Apa*I aa genotype will have an insulin resistance level of 1.21 (1.01-1.45) fold higher than women with the *Apa*I AA genotype (P=0.035). The boxplot in Figure 4.2 shows that the median of insulin resistance for aa appears to be higher than AA genotype.

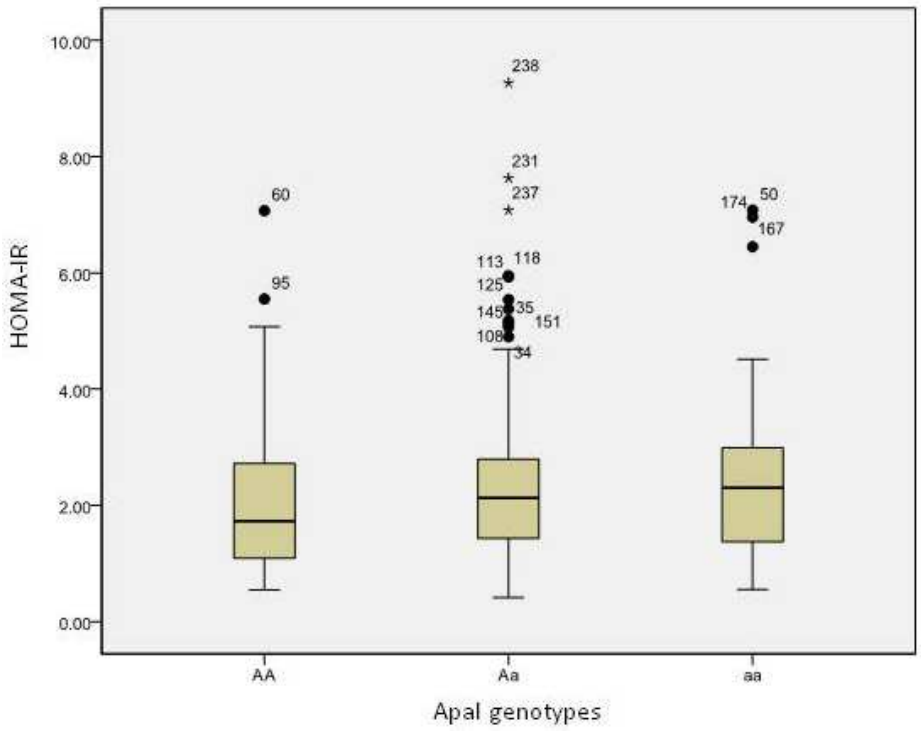


Figure 4.2. A boxplot showing the distribution of insulin resistance (HOMA-IR) scores among *Apa*I (aa, Aa and aa) genotypes in 239 women.

For *Taq*I, it is predicted that South-Asian women with the tt genotype will have insulin resistance 0.77 (0.61-0.96) fold lower than women with the TT genotype (*P*=0.022). The boxplot in Figure 4.3 shows that the median of insulin resistance for the tt appears to be lower than TT genotype.

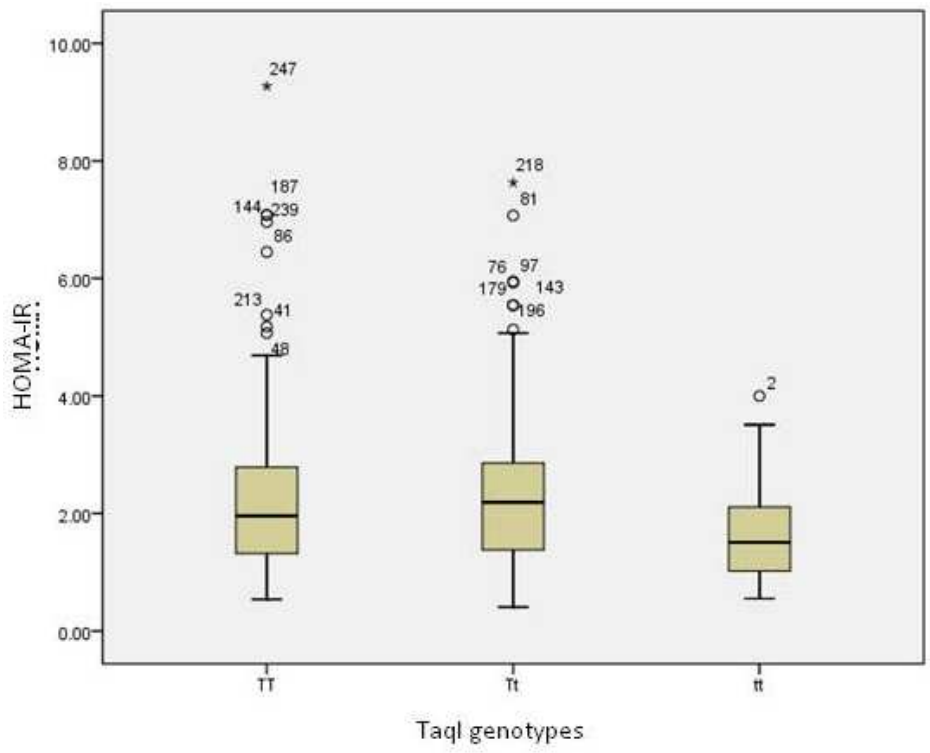


Figure 4.3. A boxplot showing the distribution of insulin resistance (HOMA-IR) scores among *Taq*I genotypes in 239 women.

Given that the b, a and T alleles and B, A and t are associated with high and low insulin resistance, respectively, in the homozygous state, it could therefore be predicted that an individual carrying haplotype genotype 'BAt-BAt' will have low insulin resistance and an individual carrying 'baT-baT' will have high insulin resistance. These predictions proved correct. An evaluation of the association between allele dose effect for the two haplotype alleles and insulin resistance was carried out (Table 4.5). Since the frequencies of other haplotype alleles were too low for investigating of the allele dose effect on insulin resistance in this cohort (Table 2.5), only the two with the highest frequencies were chosen for further analyses as biased, inaccurate and unreliable results are obtained if statistical analyses are carried out with low numbers.

Table 4.5. Prediction of insulin resistance (HOMA-IR) by *VDR* haplotype allele dose using multiple regression analysis in 239 women

Haplotype allele (n)[*]	*Copy number(n)*	*Regression coefficients (95% CI)^*	*HOMA-IR*[†]	*P*[#]
baT (214)	0[+] (77)	-	1.8 (1.5-2)	-
	1 (110)	1.12 (0.97-1.3)	2 (1.8-2.3)	0.128
	2 (52)	1.22 (1.02-1.45)	2.1 (1.8-2.4)	**0.032**
BAt (148)	0[+](113)	-	1.98 (1.8-2.2)	-
	1 (104)	0.94 (0.82-1.08)	2 (1.76-2.2)	0.369
	2 (22)	0.77 (0.61-0.97)	1.6 (1.2-2)	**0.03**

[†]HOMA-IR was BMI adjusted and was log transformed. Presented is the geometric mean (95% CI)
^Presented is the antilog of regression coefficient: the increment or decrement in HOMA-IR as compared to the control group (in brackets is the 95% CI)
Significance of differences versus the control group
[+]Control group
[*]Other haplotype alleles were not considered due to low numbers
Figures in **bold** show $P< 0.05$

It was observed that two copies of the haplotype allele baT (P=0.032) and BAt (P=0.03) were significantly associated with insulin resistance compared to no copies of their respective haplotype allele. This is substantiated by the fact that the baT-baT and BAt-BAt had the highest {2.1 (1.8-2.4)} and lowest {1.6 (1.2-2)} geometric mean of insulin resistance compared to no copies of their respective alleles (Table 4.5). It is predicted from the regression analysis that South-Asian women with two copies of the baT haplotype will have an insulin resistance level 1.22 (1.02-1.45) fold higher than women with no copy of baT (P =0.032) (Table 4.5). The boxplot in Figure 4.4 shows that individuals who had two copies of baT had higher median values of insulin resistance than no copies.

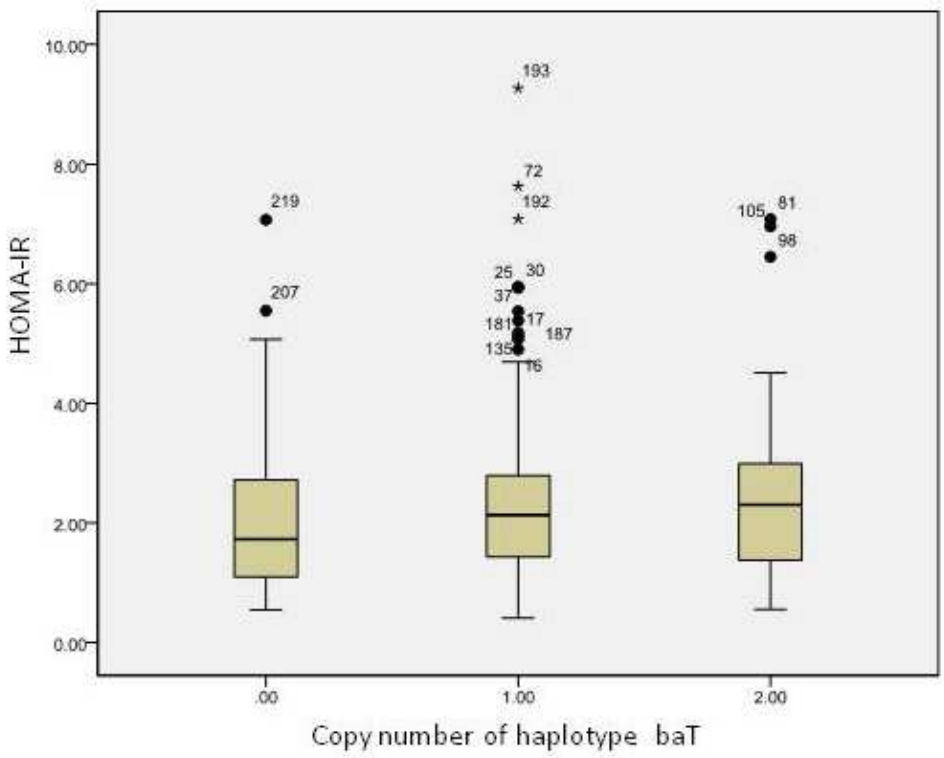


Figure 4.4. A boxplot showing the distribution of insulin resistance (HOMA-IR) scores among one, two and no copies of baT haplotype allele in 239 women.

It is also predicted that South-Asian women with two copies of BAt will have an insulin resistance level 0.77 (0.61-0.97) fold lower than women with no copies with BAt (P =0.03) (Table 4.5). The boxplot in Figure 4.5 shows that two copies of BAt has lower mean insulin resistance than no copies. This supplements the previous findings of this study as *Bsm*I bb, *Apa*I aa and *Taq*I TT were associated with high insulin resistance and BB, AA and tt were associated with low insulin resistance, respectively. Therefore baT-baT and BAt-BAt are predicted to be associated with high and low insulin resistance. However, there was no significant association between one copy of any of these haplotype alleles with insulin resistance compared to no copy of the respective alleles (P=0.128 for baT and P=0.369 for BAt).

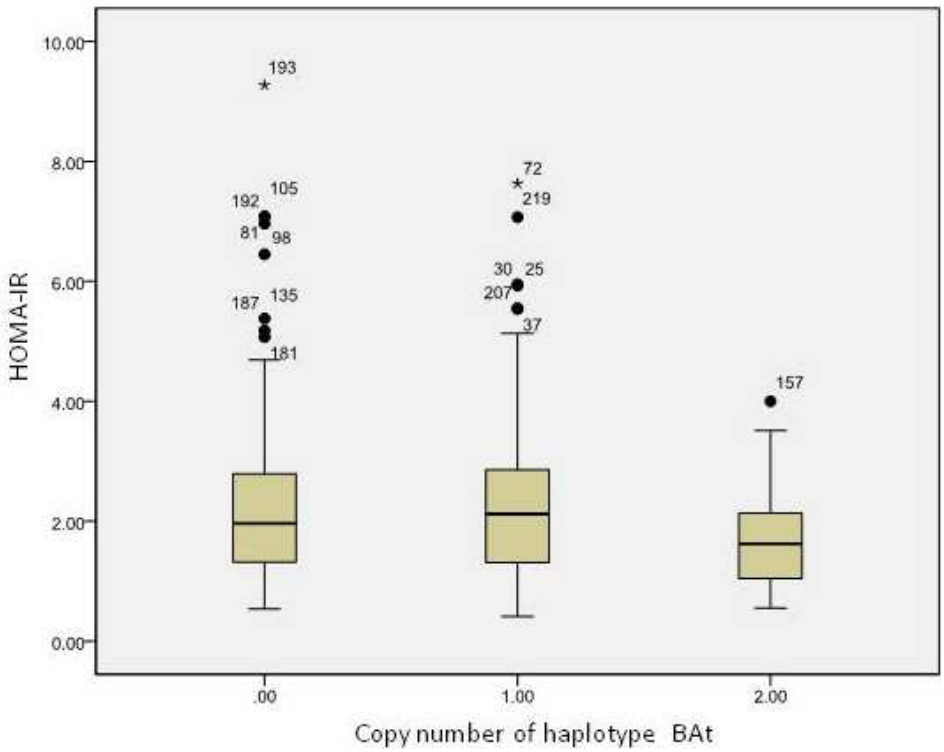


Figure 4.5. A boxplot showing the distribution of insulin resistance (HOMA-IR) scores among one, two and no copies of BAt haplotype allele in 239 women.

Associations between three haplotype genotypes (with highest frequencies) in this population with insulin resistance are shown in Table 4.6. There was no significant association between BAt-baT and insulin resistance compared to baT-baT (P =0.305) and no significant association between baT-BAT and insulin resistance compared to baT-baT (P =0.289). Only the haplotype genotypes with highest frequencies were chosen for analyses as the frequencies of other haplotype genotype among these 239 women were very low (Table 2.5). Boxplots in Figure 4.6 show the distribution of insulin resistance among all haplotype genotypes in 239 women.

Table 4.6. Prediction of insulin resistance (HOMA-IR) by *VDR* haplotype genotypes using multiple regression analysis in 239 women

Haplotype genotype (n)[*]	*Regression coefficients (95% CI)*	*HOMA-IR*[†]	*P*[#]
baT-baT (52)[+]	-	2.1 (1.8-2.4)	-
BAt-baT (64)	0.91 (0.77-1.09)	2.1 (1.8-2.4)	0.305
baT-BAT (26)	0.89 (0.71-1.11)	1.8 (1.4-2.3)	0.289

[†]HOMA-IR was BMI adjusted and was log transformed. Presented is the geometric mean (95% CI)
^Presented is the antilog of regression coefficient: the increment or decrement in HOMA-IR as compared to the control group (in brackets is the 95% CI)
Significance of differences versus the control group
[+]Control group
[*]Other haplotype genotypes were not considered due to low numbers

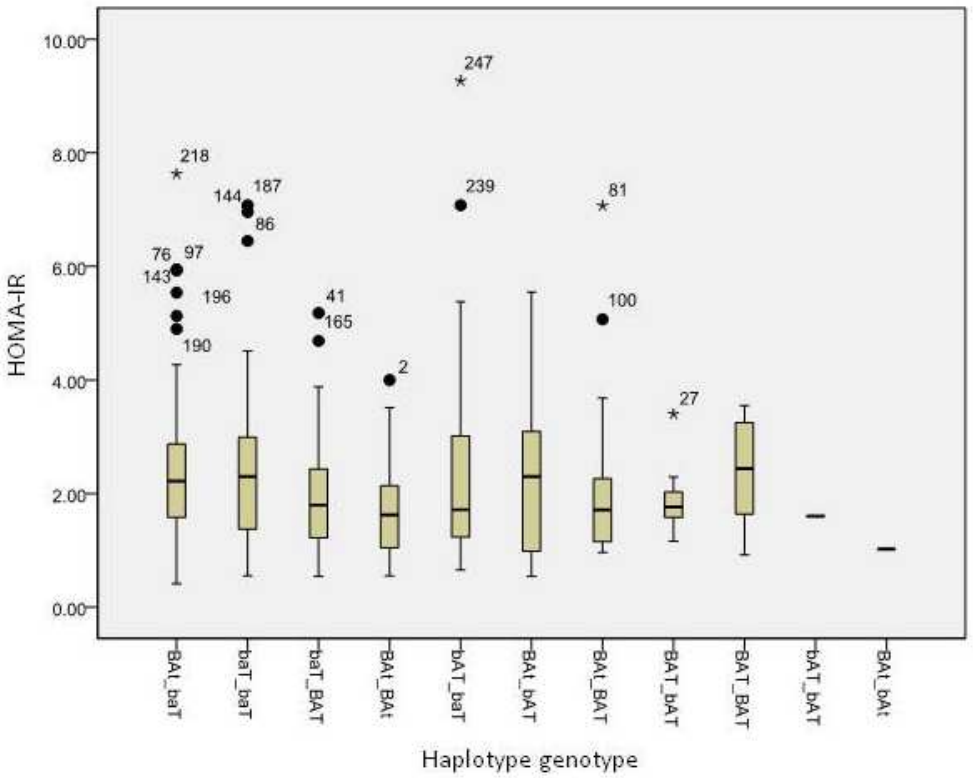


Figure 4.6. A boxplot showing distribution of insulin resistance (HOMA-IR) scores among haplotype genotypes in 239 women.

Thus, an association of insulin resistance with three SNPs of the *VDR* gene was observed. *Bsm*I bb, *Apa*I aa and *Taq*I TT genotypes were associated with high insulin resistance (HOMA-IR) as compared to BB, AA and tt genotypes. Furthermore, the baT-baT and BAt-BAt haplotype genotypes were associated with high and low insulin resistance, respectively, as compared to no copies of their respective haplotype alleles. Also, there was no association found between insulin resistance and BAt-baT and baT-BAT compared to baT-baT.

4.3.2. Results for Phase 2

The genotype distributions for the five SNPs were not significantly different between the vitamin D group and placebo group. As part of the Surya Study, it was shown that by improving the vitamin D status in the supplemented group, there was an improvement in insulin sensitivity (HOMA2%S) compared to the placebo group (Hurst, Stonehouse, & Coad, 2010). This is the reason for using HOMA2%S in the intervention group as this was already assessed as part of the Surya Study.

In order to determine whether SNPs in the *VDR* gene had any role to play in the improvement or increase in insulin sensitivity in this intervention group, various statistical tests were performed to find which group belonging to a certain genotype responded significantly different from the rest. Change in insulin sensitivity (**Δ**HOMA2%S) within a six-month period was highly skewed. Since HOMA2%S is a result of an algorithm and cannot be log transformed, non-parametric tests were used to analyse differences. No significant improvement in insulin sensitivity was expected with placebo treatment, and indeed the response in insulin sensitivity to placebo treatment did not differ significantly between any of the genotypes (Table 4.7). Even within the vitamin D group, Cdx-2 (P=0.441), *Bsm*I (P=0.395) and *Apa*I (P=0.219) did not show any significant change in insulin sensitivity.

Table 4.7. Differences in changes in insulin sensitivity (**Δ**HOMA2%S*)* over a six-month period between genotypes within vitamin D supplemented and placebo groups (Phase 2)

SNP (n)	*Placebo*		*Vitamin D group*	
	Frequency (n)	*ΔHOMA2%S^*	*Frequency (n)*	*ΔHOMA2%S^*
Cdx-2				
AA (19)	11	-2.5 (-20.6,18.8)	8	19.3(-1.1, 37.8)
AG (36)	16	-6.3 (-30.3, 11.6)	20	4.95 (-5.5, 20.3)
GG (26)	12	-7.95 (-26.4, 4.5)	14	6.4 (-4.1, 43.8)
P-value*		0.821		0.441
*Fok*I				
FF (39)	20	-2.6 (-24.5, 18.5)[a]	19	2.3 (-11.5, 10.1)
Ff (34)	15	-7.9 (-32.2, 5.8)[a]	19	29.4 (2.9, 38.1)
ff (8)	4	-5.4 (-29.3, 16.1)	4	-1.8 (-12.3, 7.3)
P-value*		0.537		**0.005**
*Bsm*I				
BB (13)	6	-14.3 (-33.1, 2.4)	7	5.4 (-18.6, 11.4)
Bb (41)	17	-16.6 (-29.6, 3.1)	24	3.4 (-3.6, 32.7)
bb (27)	16	-1.5 (-7.8, 18.8)	11	20 (-2.5, 29.4)
P-value*		0.263		0.395
*Apa*I				
AA (21)	9	-20.6 (-37.6, 8.1)	12	1.8 (-13.2, 11.1)
Aa (41)	19	-2.6 (-23, 5.8)	22	4.05 (-2.5, 37.1)
aa (19)	11	-2.3 (-25, 18.8)	8	20.2 (-0.2, 28.2)
P-value*		0.412		0.219
*Taq*I				
TT (40)	23	-5.9 (-32.2, 17.4)	17	11.4 (-3.4, 31.3)
Tt (36)	14	-2.9 (-20.8, 7.8)[b]	22	4.1 (-1.8, 32.7)
tt (5)	2	-28.2 (-35.8, N.A.)[b]	3	-18.6 (-48.6, N.A.)
P-value*		0.366		**0.024**

^ change in HOMA2%S expressed as median (25^{th}, 75^{th} percentile)

a, b: different superscripts indicate significant differences within genotypes ($P<0.016$ after Bonferroni adjustment). However, post hoc analysis for *Taq*I between Tt and tt could not be considered due to extremely low frequency of tt ($n=5$).

*as determined by Kruskal-Wallis test

Figures in **bold** show $P<0.05$

However, the change in insulin sensitivity among *Fok*I (P=0.005) and *Taq*I (P=0.024) genotypes were significantly different in the vitamin D group but not in placebo group, as expected (P=0.537 for *Fok*I and P=0.366 for *Taq*I) (Table 4.7). Post-hoc analyses of *Fok*I and *Taq*I genotypes in the vitamin D supplemented group showed that significant differences lay in the change in insulin sensitivity between FF and Ff genotypes only (P<0.016), and not between TT and Tt genotypes (P>0.016) after Bonferroni adjustments (Table 4.7). The median for change in insulin sensitivity in the vitamin D group for Ff {29.4 (2.9, 38.1)} was much higher than FF {2.3 (-11.5, 10.1)} (Table 4.7). Figure 4.7 shows the distribution of **Δ**HOMA2%S among genotypes of *Fok*I between placebo and vitamin D groups, respectively. It is evident that the improvement in insulin sensitivity was quite high for *Fok*I Ff genotype compared with FF and ff genotypes in the vitamin D group. Also, the difference between change in insulin sensitivity for *Fok*I Ff genotype between placebo and vitamin D group appears to be the largest as compared to FF and ff. It is predicted that women carrying *Fok*I Ff will have a greater change in insulin sensitivity with vitamin D supplements as compared to placebo group with the same genotype and women carrying *Fok*I FF genotype.

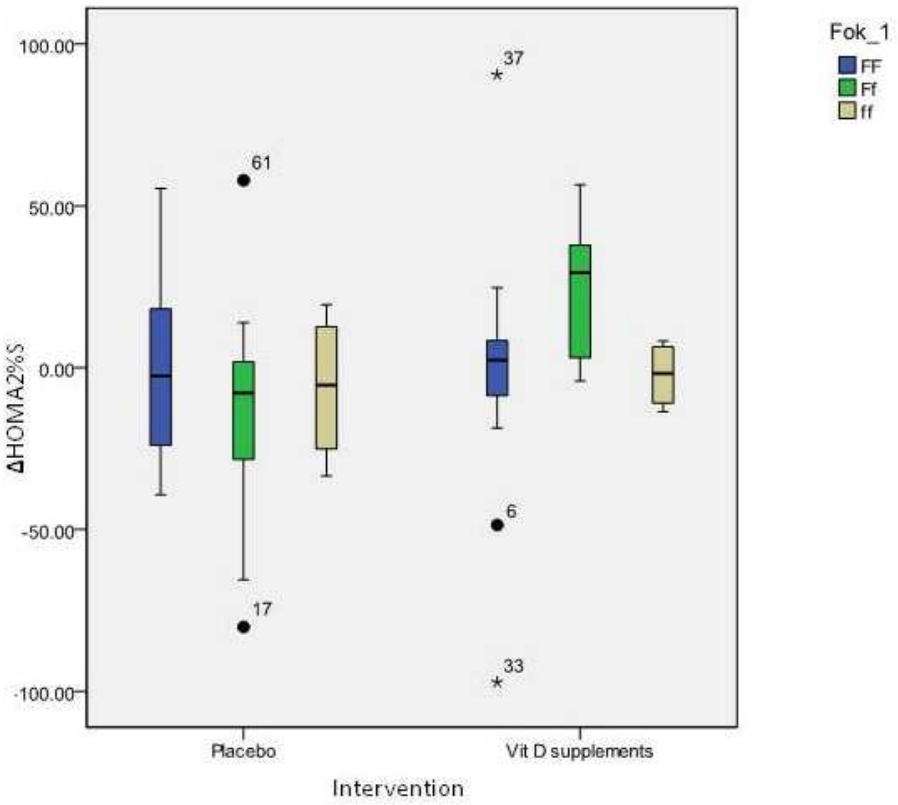


Figure 4.7. A boxplot showing the distribution of change in insulin sensitivity (ΔHOMA2%S) among *Fok*I genotypes (FF, Ff and ff) between placebo and vitamin D supplemented groups.

When further post hoc analyses were carried out for the rest of the genotypes of the *Taq*I, significant differences were observed between Tt and tt (P<0.016) but not between TT and tt; TT and Tt genotypes. However, this significant difference was not considered because the numbers of *Taq*I tt (n=5) participants were very low (Table 4.7). Since the participants were matched according to age and BMI, these two confounding factors were not considered while analysing. Although Cdx-2 AA, *Bsm*I bb, *Apa*I aa and *Taq*I TT failed to reach significance in the vitamin D supplemented group, there seems to be a trend towards having a greater increase in insulin sensitivity (Table 4.7). Figures 4.8, 4.9, 4.10 and 4.11 show that the change in insulin sensitivity for Cdx-2 AA, *Bsm*I bb, *Apa*I aa and *Taq*I TT is large compared to other genotypes and with respect to their placebo groups.

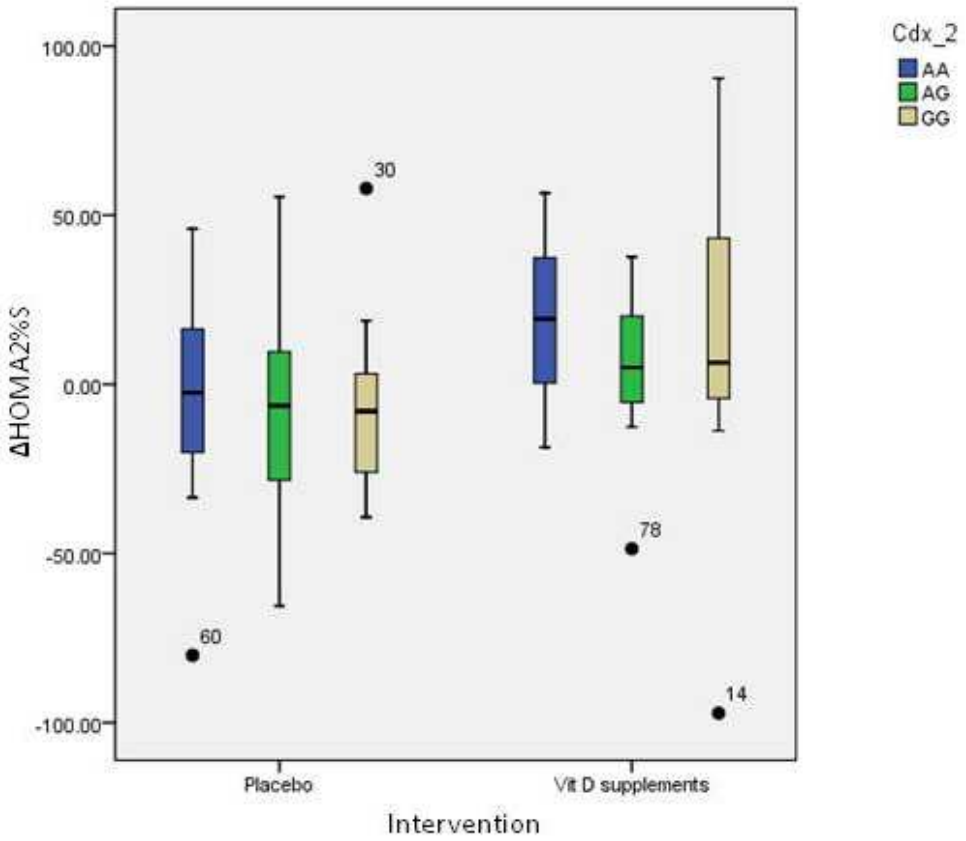


Figure 4.8. A boxplot showing the distribution of change in insulin sensitivity (ΔHOMA2%S) among Cdx-2 genotypes (AA, AG and GG) between placebo and vitamin D supplemented groups.

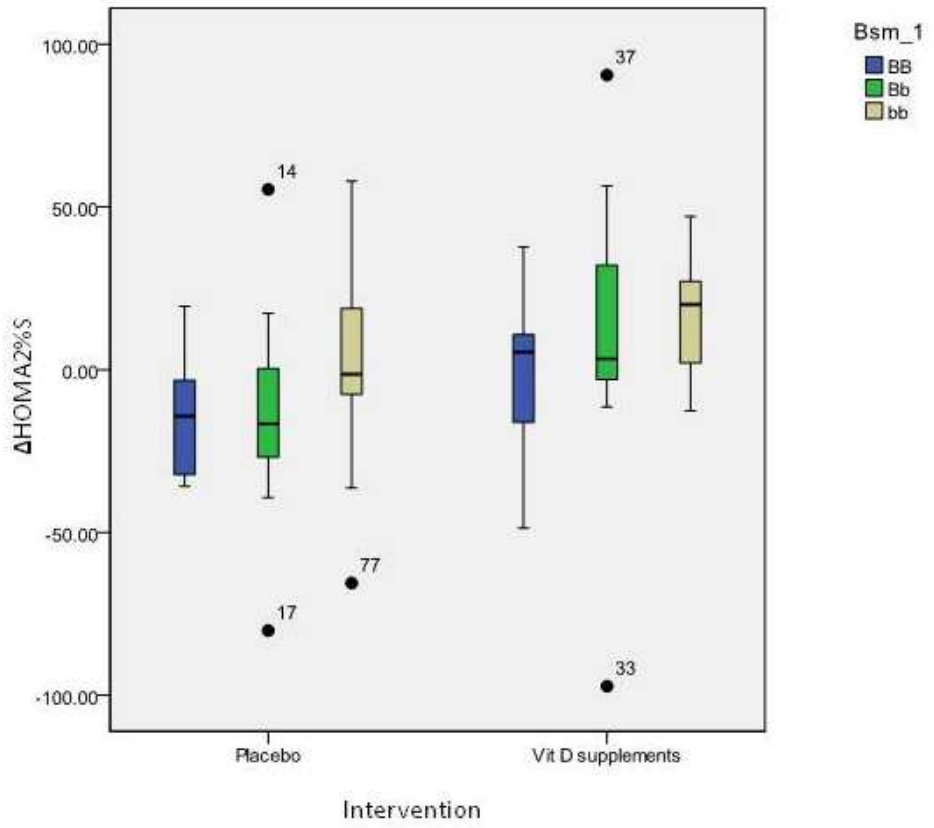


Figure 4.9. A boxplot showing the distribution of change in insulin sensitivity (ΔHOMA2%S) among *Bsm*I genotypes (BB, Bb and bb) between placebo and vitamin D supplemented groups.

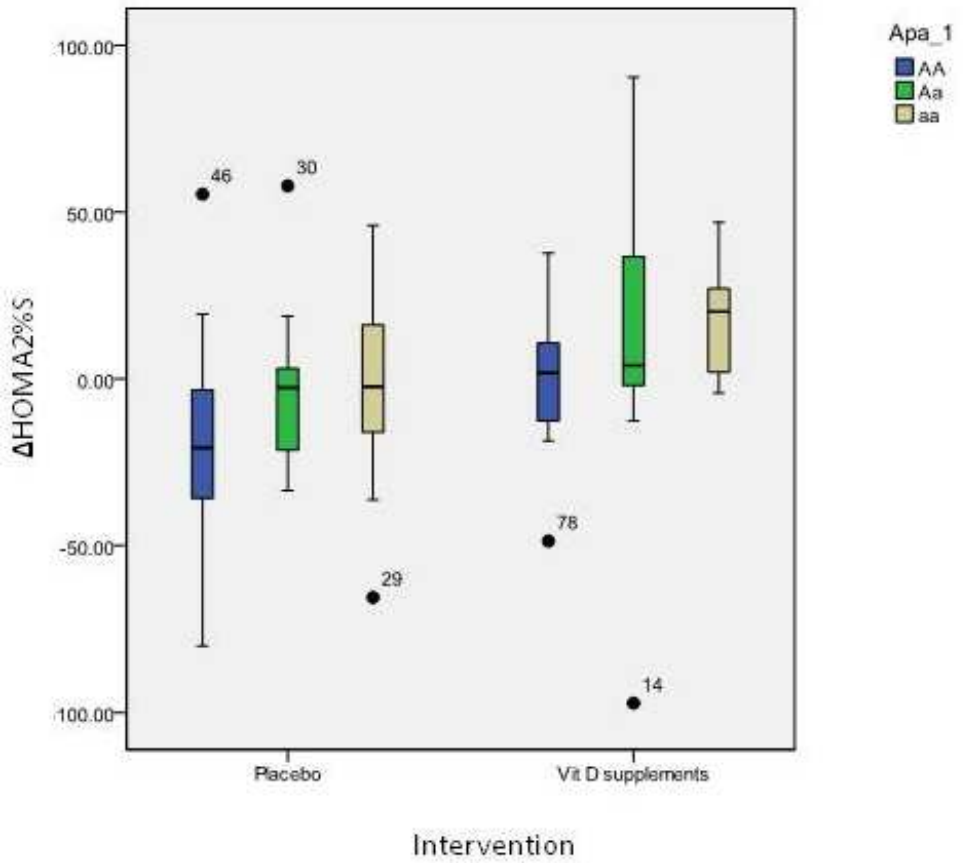


Figure 4.10. A boxplot showing the distribution of change in insulin sensitivity (ΔHOMA2%S) among *Apa*I genotypes (AA, Aa and aa) between placebo and vitamin D supplemented groups

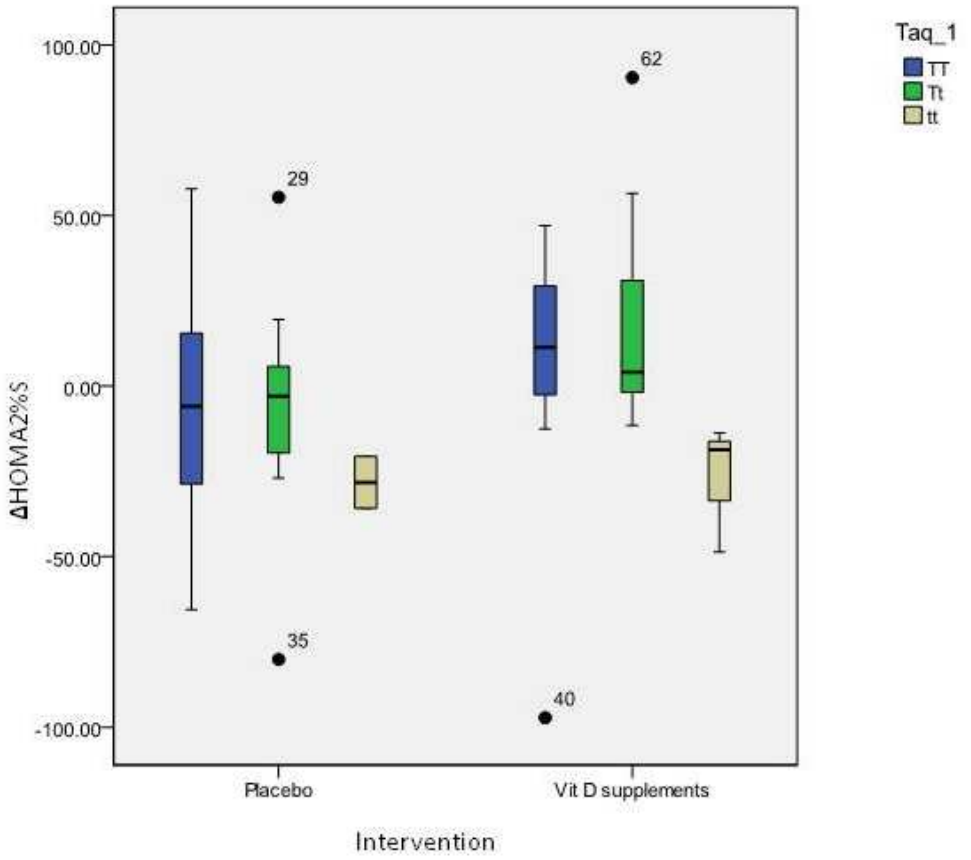


Figure 4.11. A boxplot showing the distribution of change in insulin sensitivity (ΔHOMA2%S) among *Taq*I genotypes (TT, Tt and tt) between placebo and vitamin D supplemented groups

The effect of haplotype allele dose and haplotype genotype on the response to this intervention in vitamin D and placebo groups could not be assessed as the frequencies were too low (Tables 4.8, 4.9).

Table 4.8. Distribution of haplotype allele in the intervention group

Haplotype allele (n)	*Copy number*	*Intervention*	
		Placebo	*Vitamin D supplements*
baT (79)		41	38
	0	9	12
	1	19	22
	2	11	8
BAt (46)		18	28
	0	23	17
	1	14	22
	2	2	3
BAT (21)		11	10
	0	29	34
	1	9	6
	2	1	2
bAT (16)		8	8
	0	31	34
	1	8	8
	2	0	0

Table 4.9. Distribution of haplotype genotype in the intervention group

Haplotype genotype (n)	*Intervention*	
	Placebo	*Vitamin D supplements*
BAt- baT (24)	9	15
baT- baT (19)	11	8
baT- BAT (9)	5	4
BAt- BAt (5)	2	3
bAT- baT (8)	5	3
BAt- bAT (7)	2	5
BAt- BAT (5)	3	2
BAT- bAT (1)	1	0
BAT- BAT (3)	1	2

4.4. Discussion

The purpose of this study was twofold. The first aim was to investigate whether *VDR* genotype contributed to the determination of insulin resistance when BMI was adjusted in multiple regression. The other aim was to investigate the change in insulin sensitivity in a six month intervention with respect to *VDR* genotypes under the influence of vitamin D supplements. For the first aim, this study showed an association of *VDR* genotype with insulin resistance in 239 women. *Bsm*I BB, *Apa*I AA and *Taq*I tt genotypes were significantly associated with lower insulin resistance (HOMA-IR) levels compared to bb, aa and TT genotypes. Furthermore, the geometric mean of insulin resistance for BB, AA and tt were the lowest compared to other genotypes. The results are partly in agreement with the findings of Oh, and Barrett-Connor (2002) where *Bsm*I bb genotype was associated with high insulin resistance. In addition to *Bsm*I, the study here finds an association of *Apa*I and *Taq*I with insulin resistance, which was not found by Oh, and Barrett-Connor. Very few studies have been conducted in a non-diabetic population looking at association between *VDR* genotypes and insulin resistance.

These results were further supplemented by the results obtained for allele dose effect. The haplotyping method adapted from Uitterlinden et al. (1996) allowed five haplotype alleles to be discriminated and their combinations led to twelve different haplotype genotypes. However, the frequencies of these alleles and genotypes were very low and so only the two alleles and three genotypes with highest frequencies were analysed for their association with insulin resistance. When allele dose of baT and BAt was analysed, it was found that two copies of both alleles had a significant effect on insulin resistance compared to no copies of their respective alleles. The former (baT-baT) was associated with higher insulin resistance while the latter (BAt-BAt) with lower insulin resistance. This supplements the findings as *Bsm*I bb, *Apa*I aa and *Taq*I tt genotypes were again found to be associated with higher insulin resistance or in other words, BB, AA and tt genotypes were found to be associated with lower insulin resistance.

No significant associations were found between haplotype genotypes and insulin resistance when baT-baT was the control group; the geometric means of insulin resistance for all three

genotypes were quite close. This is in agreement with the allele dose results as the geometric mean of insulin resistance for one copy of baT {2 (1.8-2.3)} and two copies of baT {2.1 (1.8-2.4)} were very close and so no significant difference was found when haplotype genotypes were compared against each other.

When the second aim was assessed, it was found that the change in insulin sensitivity (calculated by HOMA2%S) among the *Fok*I and *Taq*I SNPs was significantly different compared to other genotypes. Furthermore, the difference in change in insulin sensitivity between the placebo and vitamin D supplemented group with the *Fok*I Ff genotype was large, which showed that there was significant improvement in insulin sensitivity in individuals who had Ff genotype in response to vitamin D supplements. Also, the change in insulin sensitivity for Ff genotype was significantly different from FF genotype. This meant that women carrying *Fok*I Ff genotype responded significantly different from women carring the FF genotype under vitamin D supplementation and it seemed that this change in insulin sensitivity was much larger for Ff than FF genotype. The change in insulin sensitivity was also significantly different with the *Taq*I genotypes in vitamin D supplemented group but further analyses to compare *Taq*I genotypes showed that there was no significant difference between the groups except Tt and tt genotypes. However, the significant difference between Tt and tt genotypes with respect to change in insulin sensitivity could not be considered as the frequency for tt genotype was very low. Low frequency of *Taq*I tt genotype affects the statistical analyses and so the significant difference observed is not very reliable. Nevertheless, a trend was observed for *Taq*I TT along with Cdx-2 AA, *Bsm*I bb, *Apa*I aa having a greater change in insulin sensitivity with vitamin D supplementation. This is not surprising as the results from phase 1 showed that individuals with *Bsm*I bb, *Apa*I aa and *Taq* TT genotypes had higher insulin resistance (thus lower insulin sensitivity) than BB, AA and tt genotypes. The lower insulin sensitivity in bb, aa and TT individuals then resulted in a greater change in insulin sensitivity as compared to BB, AA and tt with vitamin D supplementation since their baseline insulin sensitivity values were already low at the start of the intervention. Therefore, the lower insulin resistance (higher insulin sensitivity) group in phase 1 (BB, AA and tt) would be expected to have a smaller increase in insulin sensitivity due to vitamin D supplementation. The high values of change in insulin sensitivity in response to vitamin D treatment with Cdx-2 AA

and *Fok*I Ff genotypes could have been due to an overestimation of the magnitude of the association due to low numbers. At the same time, one cannot exclude the possibility that the response to treatment could be significantly different for the five SNPs. However, a study with a larger cohort of individuals with the same study outline for phase 2 may increase the number of individuals per genotype group considerably, which may help to achieve stable and precise point estimates. Another reason for low numbers of certain genotypes (*Bsm*I BB, *Apa*I AA and *Taq*I tt) and hapotypes (for example BAt-BAt) apart from the sample size was that phase 2 included women who had high insulin resistance (HOMA-IR>1.93). Since BB, AA and tt genotypes were found to be associated with low insulin resistance, fewer individuals were included having these genotypes and haplotype in phase 2.

Even though there was an association found in 239 women between insulin resistance with 3' end polymorphisms of the *VDR* gene, as far as the VDR protein structure is concerned, *Bsm*I-*Apa*I-*Taq*I SNPs are not likely to be functional but they are still useful in association studies as they can be used as markers. *Bsm*I and *Apa*I SNPs are present in intron 8 and *Taq*I SNP which is present in exon 9, is a synonymous polymorphism that does not change the sequence of amino acid. However, *Taq*I TT was shown to be associated with high VDR mRNA and protein levels (Ogunkolade et al., 2002). When an association is found between a disease and a marker, it is believed that the association is in fact with a truly functional allele which is present elsewhere but is linked to the marker allele. However, the degree of this association depends on the extent and strength of LD between the marker allele and disease allele (Uitterlinden el al., 2004). Also, interactions among genes and gene environment may also influence this association between the gene and disease (Kim et al., 2001).

An association of baT-baT and BAt-BAt haplotype genotype with higher and lower insulin resistance, respectively, could be due to linkage disequilibrium of larger number of 'A's in the polyA VNTR at the 3' end with baT haplotype allele while the BAt is linked to a smaller number of A's (*n*=13-17) (Morrison et al., 1994). Uitterlinden et al. (2004) stated that the BAt haplotype allele might confer a better response than baT to treatment to calcitriol, calcium and corticosteroids after they analysed various studies focussed on

responses involving changes in BMD. This might mean that BAt together with a smaller number of 'A's could confer slightly better mRNA stability and half-life resulting in higher levels of VDR protein being present in the target cell. In the present study, this might give the target cell with BAt a better response to vitamin D, thus decreasing insulin resistance. This could be the reason for the association of lower insulin resistance with two copies of the BAt haplotype allele compared to no copy and higher insulin resistance with baT-baT. However, another study by Whitfield et al., (2001) demonstrated functional significance of *Fok*I genotype (F and f) and the poly(A) stretch in the 3' UTR with long (L) and short (S) alleles. They reported that when both polymorphic sites are considered simultaneously, the genotype FL was associated with high VDR activity and fS genotype with low activity. This association was not evident when the polymorphisms were analysed separately. One of the explanations they hypothesised was that the L allele might produce VDR mRNA that was more stable and/or was translated more efficiently into VDR protein than the S allele. However, a major drawback of this study was that it was an *in vitro* study and they studied twenty fibroblast cell lines of different *VDR* genotypes. The cell culture conditions may not always mimic the *in vivo* environment and so the true observations may not be evident under the conditions of the experiment. In any case, these studies illustrate the importance of analysing multiple polymorphisms simultaneously which could not have been predicted by analysing single SNPs. This approach was the strength of the present research where 3' end polymorphisms were analysed together. It has been demonstrated that in addition to genotypes there was an association of insulin resistance with haplotypes. However, if haplotyping could be performed for all five polymorphisms, then one could study the effect of different combinations of polymorphisms on insulin resistance. For example, it would be intriguing to examine the effect of a haplotype consisting of Cdx-2 A (which is known for higher production of VDR mRNA in intestinal cell than G), *Fok*I F (known to produce more active VDR protein) and baT (supposedly known for more active/less unstable 3' UTR resulting in more VDR protein being produced) on insulin resistance.

The responsiveness of a subset of this cohort which involved measuring changes in insulin sensitivity to vitamin D treatment, depended on the genotype each individual possessed. Individuals with Ff showed significantly greater change in insulin resistance than FF, and a trend towards greater increase in insulin sensitivity (though not significant) was observed

for individuals carrying Cdx-2 AA, *Apa*I aa, *Bsm*I bb, and *Taq*I TT genotypes compared to other genotypes.

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Chapter 5
General Discussion

Despite the growing knowledge investigating the associations between *VDR* polymorphisms and insulin resistance; and vitamin D supplementation and insulin resistance, this remains the first study to investigate responsiveness of *VDR* gene polymorphisms to insulin sensitivity under vitamin D supplementation.

Reports have been published demonstrating the positive effects of vitamin D supplementation on insulin resistance in either type 2 diabetic patients or healthy but insulin resistant individuals. Similarly, genetic predisposition of certain ethnic groups to insulin resistance has been linked to polymorphisms in the *VDR* gene. Since vitamin D exerts its biological effects through VDR, the latter is suspected to affect most of the functions and downstream proteins in the vitamin D pathway. The primary objective of this study was to investigate the association between *VDR* SNPs with disease conditions and intervention responsiveness which included insulin sensitivity/ resistance. This study affirms the association of *VDR* polymorphisms with insulin resistance. This showed a possibility of genetic susceptibility of this population to insulin resistance and consequently a high risk of developing type 2 diabetes.

The efficiency of vitamin D action may be dependent on the form of VDR protein present. This in turn may be dependent on *VDR* genotypes an individual possesses which may influence VDR binding with vitamin D (Ogunkolade et al., 2002). This will affect all the downstream protein in the vitamin D pathway including secretion of insulin by the pancreas and responsiveness to insulin by the cells of the body, giving rise to insulin resistance. However, this study along with few others showed that insulin resistance can be improved by vitamin D supplementation. Therefore, before recommendations for the dose and duration of vitamin D supplementation, it is important that there is a clear understanding of the size of the dose required that suits the genetic profile of the individual. This is the essence of personalised medicine. For example, this study showed that an individual who has the Ff genotype for the *Fok*I SNP may be expected to respond better to vitamin D supplements as compared to another individual who has ff or FF with the same dose of vitamin D supplements for the same duration. Thus, if we can identify genotypes that are more responsive to treatment with vitamin D, such treatment may only be given to those expected to respond rather than to all. This in turn would lead to more efficient use of the

public health dollar. However, exposure to sun is a much cheaper option but a consensus on safe exposure of sunlight to prevent skin cancer needs to be formulated.

Further studies involving genetic association assessment are needed to demonstrate the functional effects of *VDR* polymorphisms on disease development involving a larger cohort of population across different ethnicities. Research has reported that certain population groups are more susceptible to phenotypes related to hypovitaminosis D than other groups of different ethnicity. Therefore, large cohorts of populations need to be investigated for association of disease phenotype and SNPs of the *VDR* gene across different ethnicities. This could explain the inconsistency of results across various association studies.

Insulin sensitivity improved over a period of six months with 4000 IU/d during the Surya Study. However, the improvement was seen only after six months and only when the serum 25(OH)D concentration reached 80 nmol/L in the intervention group. Even though there was an improvement observed after six months, each individual responded differently according to the genotype they possessed. An investigation of long-term duration and higher dose of vitamin D supplementation on insulin resistance and vitamin D deficient individuals should be conducted with a larger cohort of women to better understand their responsiveness to vitamin D treatment. The small sample size of eighty-one in the intervention was a limitation of this study. This lowered the frequency of haplotypes and genotypes which affected the statistical analyses. While *Taq*I and *Fok*I SNPs may play a direct role in insulin resistance, the non-association of the other polymorphisms (Cdx-2*,* *Bsm*I and *Apa*I) could be explored further with a larger cohort of subjects.

It is recommended that the focus on anonymous polymorphisms at the 3' end and their associations with disease phenotype be moved to study other polymorphisms throughout the gene that potentially have greater functional effects on protein structure.

This study also compared the two techniques, qPCR and RFLP-PCR, for analysis of SNPs at the 3' end of the *VDR* gene and demonstrated that the latter technique was a much more reliable and lower cost methodology for SNP genotyping. Further, RFLP-PCR allowed identification of haplotypes which was not possible by qPCR. Future research may include validation of a new robust, simple, rapid and cost-effective method called the tetra-primer

amplification refractory mutation system-PCR (tetra-primer ARMS-PCR) with the traditional RFLP-PCR for analysis of SNPs. The former method employs the use of two pairs of primers to amplify two different alleles of a SNP. These allele-specific primers have a mismatch at the 3' end to match with the specific allele. In order to further increase the specificity, a deliberate mismatch at -2 position from 3' terminus is incorporated in both inner primers (Okayama et al., 2004). This is very similar to the method used for Cdx-2 genotyping (ASM-PCR) except that the specificity is enhanced here by a second deliberate mismatch at position -2 from the 3'-terminus. There is no post PCR manipulation such as digestion with restriction enzymes as for RFLP-PCR and so it would be cheaper and quicker than the classical technique (Ye, Dhillon, Ke, Collins, & Day, 2001).

This study highlighted the association of SNPs and insulin resistance but the biochemical pathway involving vitamin D, proteins in the insulin signalling pathway and VDR protein influenced by *VDR* polymorphisms is unclear. It is worth investigating the relationship and interaction between the three. Several other proteins and genes are involved in the metabolism and action of vitamin D. Examples are *Gc, CYP27B1* and *CYP24A1* that encode for vitamin D-binding protein, 1,α (OH)ase and 24-hydroxylase, respectively. These are involved in the vitamin D metabolism and could be investigated for their role in insulin resistance.

Based on the results obtained, the hypothesis stating that qPCR is much reliable technique for genotyping the 3' end of the *VDR* gene than RFLP-PCR was not supported. However, the second hypothesis that there is an association of *VDR* gene polymorphisms with insulin resistance and that each individual will respond differently to insulin sensitivity as per their genotype under vitamin D treatment was supported.

References

Almasan, A., Mangelsdorf, D. J., Ong, E. S., Wahl, G. M., & Evans, R. M. (1994). Chromosomal localization of the human retinoid X receptors. *Genomics, 20*(3), 397-403.

Arai, H., Miyamoto, K. I., Taketani, Y., Yamamoto, H., Iemori, Y., Morita, K., et al. (1997). A vitamin D receptor gene polymorphism in the translation initiation codon: Effect on protein activity and relation to bone mineral density in Japanese women. *Journal of Bone and Mineral Research, 12*(6), 915-921.

Arai, H., Miyamoto, K. I., Yoshida, M., Yamamoto, H., Taketani, Y., Morita, K., et al. (2001). The polymorphism in the caudal-related homeodomain protein Cdx-2 binding element in the human vitamin D receptor gene. *Journal of Bone and Mineral Research, 16*(7), 1256-1264.

Armas, L. A. G., Hollis, B. W., & Heaney, R. P. (2004). Vitamin D_2 is much less effective than vitamin D3 in humans. *The Journal of Clinical Endocrinology and Metabolism, 89*(11), 5387 - 5391.

Arnaud, J., & Constans, J. (1993). Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). *Human Genetics, 92*(2), 183-188

Bai, Y., Yu, Y., Yu, B., Ge, J., Ji, J., Lu, H., et al. (2009). Association of vitamin D receptor polymorphisms with the risk of prostate cancer in the Han population of Southern China. *BMC Medical Genetics, 10*.

Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., et al. (1988). Cloning and Expression of Full-Length cDNA Encoding Human Vitamin D Receptor. *Proceedings of the National Academy of Sciences , USA, 85*(10), 3294 - 3298.

Bakwin, H., & Bakwin, R. M. (1933). Vitamin D deficiency. Tetany in infants without rickets. *The Journal of Pediatrics, 3*(6), 880-882

Barone, A., Giusti, A., Pioli, G., Girasole, G., Razzano, M., Pizzonia, M., et al. (2007). Secondary hyperparathyroidism due to hypovitaminosis D affects bone mineral density response to Alendronate in elderly women with osteoporosis: A randomized controlled trial. *Journal of the American Geriatrics Society, 55*(5), 752–757.

Basic Local Alignment Search Tool. Retrieved April 16, 2009, from http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9606

Barr, R. J., Stewart, A., Torgerson, D. J., & Reid, D. M. (2010). Population screening for osteoporosis risk: a randomised control trial of medication use and fracture risk. *Osteoporosis International, 21*(4), 561-568.

Berndt, S. I., Dodson, J. L., Huang, W. S., & Nicodemus, K. K. (2006). A systematic review of vitamin D receptor gene polymorphisms and prostate cancer risk. *The Journal of Urology, 175*(5), 1613-1623

Bid, H. K., Konwar, R., Aggarwal, C. G., Gautam, S., Saxena, M., Nayak, V. L., et al. (2009). Vitamin D receptor (FokI, BsmI and TaqI) gene polymorphisms and type 2 diabetes mellitus: A North Indian study. *Indian Journal of Medical Sciences, 63*(5), 187-194.

Bid, H. K., Mishra, D. K., & Mittal, R. D. (2005). Vitamin-D receptor (VDR) gene (Fok-I, Taq-I and Apa-I) polymorphisms in healthy individuals from north Indian population. *Asian Pacific Journal of Cancer Prevention 6*(2), 147-152.

Bikle, D. D. (2009a). Extra renal synthesis of 1, 25-dihydroxyvitamin D and its health implications. *Clinical Reviews in Bone and Mineral Metabolism, 7*, 114-125.

Bikle, D. D. (2009b). Nonclassic actions of vitamin D. *The Journal of Clinical Endocrinology and Metabolism, 94*(1), 26-34.

Bikle, D. D. (2010). Vitamin D: newly discovered actions require reconsideration of physiologic requirements. *Trends in Endocrinology and Metabolism, 638*, 1-10.

Borissova, A. M., Tankova, T. S. V., Kirilov, G. G., Dakovska, L. N., & Kovacheva, R. (2003). The effect of vitamin D3 on insulin secretion and peripheral insulin sensitivity in type 2 diabetic patients. *International Journal of Clinical Practice, 57*(4), 258-261.

Botstein, D., White, R. L., Skolnick, M., & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics, 32*(3), 314-331.

Boucher, B. J., Mannan, N., Noonan, K., Hales, C. N., & Evans, S. J. W. (1995). Glucose intolerance and impairment of insulin secretion in relation to vitamin D deficiency in East London Asians. *Diabetologia, 38*(10), 1239-1245.

Canaff, L., & Hendy, G. N. (2002). Human calcium-sensing receptor gene: Vitamin D response elements in promoters P1 and P2 confer transcriptional responsiveness to 1, 25-dihydroxyvitamin D. *The Journal of Biological Chemistry, 277*(33), 30337 - 30350.

Carlberg, C., & Seuter, S. (2009). A genomic perspective on vitamin D signaling. *Anticancer Research, 29*(9), 3485-3494.

Cavalier, E., Delanaye, P., Chapelle, J.-P., & Souberbielle, J.-C. (2009). Vitamin D: current status and perspectives. *Clinical Chemistry and Laboratory Medicine, 47*(2), 120-127.

Chang, J. C., & Kan, Y. W. (1981). Antenatal diagnosis of sickle cell anaemia by direct analysis of the sickle mutation. *The Lancet, 2*(8256), 1127-1129.

Chemistry of Vitamin D (1999). Retrieved October 28, 2009, from http://vitamind.ucr.edu/chem.html

Chesney, R. W. (1989). Vitamin D: Can an upper limit be defined? *The Journal of Nutrition, 119*(12), 1825 -1828.

Chiu, K. C., Chu, A., Go, V. L. W., & Saad, M. F. (2004). Hypovitaminosis D is associated with insulin resistance and β cell dysfunction. *The American Journal of Clinical Nutrition, 79*(5), 820-825.

Chiu, K. C., Chuang, L.-M., & Yoon, C. (2001). The vitamin D receptor polymorphism in the translation initiation codon is a risk factor for insulin resistance in glucose tolerant Caucasians. *BMC Medical Genetics, 2.*

Crawford, D. C., & Nickerson, D. A. (2005). Definition and clinical importance of haplotypes. *Annual Review of Medicine, 56*, 303-320.

Crofts, L. A., Hancock, M. S., Morrison, N. A., & Eisman, J. A. (1998). Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proceedings of the National Academy of Sciences of the United States of America, 95*(18), 10529-10534.

Davies, P. S. W., Bates, C. J., Cole, T. J., Prentice, A., & Clarke, P. C. (1999). Vitamin D: Seasonal and regional differences in preschool children in Great Britain. *European Journal of Clinical Nutrition, 53*, 195-198.

Davis, C. D. (2008). Vitamin D and cancer: Current dilemmas and future research needs. *The American Journal of Clinical Nutrition, 88*(2), 565S-569S.

Deepa, R., Shanthirani, C. S., Premalatha, G., Sastry, N. G., & Mohan, V. (2001). Prevalence of insulin resistance syndrome in a selected south Indian population - The Chennai urban population study-7 [CUPS-7]. *Indian Journal of Medical Research, 115*, 118-127.

Demay, M. B., Kiernan, M. S., DeLuca, H. F., & Kronenberg, H. M. (1992). Sequences in the human parathyroid hormone gene that bind the 1, 25-dihydroxyvitamin D3 receptor and mediate transcriptional repression in response to 1, 25-dihydroxyvitamin D3. *Proceedings of the National Academy of Sciences, USA, 89*(17), 8097-8101.

Dickinson, J. L., Perera, D. I., Mei, A. F. v. d., Ponsonby, A.-L., Polanowski, A. M., Thomson, R. J., et al. (2009). Past environmental sun exposure and risk of multiple sclerosis: A role for the Cdx-2 Vitamin D receptor variant in this interaction. *Multiple Sclerosis 15*(5), 563-570.

Drummond, A. J., Ashton, B., Cheung, M., Heled, J., Kearse, M., Moir, R., et al. (2009). *Geneious v.4.7*. Retrieved 23 April, 2010, from www.geneious.com/

Eccleshall, T. R., Garnero, P., Gross, C., Delmas, P. D., & Feldman, D. L. (1998). Lack of correlation between start codon polymorphism of the vitamin D receptor gene and bone mineral density in premenopausal french women: The OFELY study. *Journal of Bone and Mineral Research, 13*(1), 31-35.

Ezura, Y., Nakajima, T., Kajita, M., Ishida, R., Inoue, S., Yoshida, H., et al. (2003). Association of molecular variants, haplotypes, and linkage disequilibrium within the human vitamin D-binding protein (DBP) gene with postmenopausal bone mineral density. *Journal of Bone and Mineral Research, 18*(9), 1642-1649.

Fang, Y., Meurs, J. B. J. v., Bergink, A. P., Hofman, A., Duijn, C. M. v., Leeuwen, J. P. v., et al. (2003). Cdx-2 polymorphism in the promoter region of the human vitamin D receptor gene determines susceptibility to fracture in the elderly. *Journal of Bone and Mineral Research, 18*(9), 1632-1641.

Fang, Y., Meurs, J. B. J. v., d'Alesio, A., Jhamai, M., Zhao, H., Rivadeneira, F., et al. (2005). Promoter and 3′-untranslated-region haplotypes in the vitamin D receptor gene predispose to osteoporotic fracture: The Rotterdam study. *American Journal of Human Genetics, 77*(5), 807–823.

Fang, Y., Meurs, J. B. J. v., Rivadeneira, F., Schoor, N. M. v., Leeuwen, J. P. T. v., Lips, P., et al. (2007). Vitamin D receptor gene haplotype is associated with body height and bone size. *The Journal of Clinical Endocrinology and Metabolism, 92*(4), 1491-1501.

Fang, Y., Rivadeneira, F., Meurs, J. B. J. v., Pols, H. A. P., Ioannidis, J. P. A., & Uitterlindena, A. G. (2006). Vitamin D receptor gene BsmI and TaqI polymorphisms and fracture risk: A meta-analysis. *Bone 39*(4), 938-945.

Ferrannini, E. L. E. (2006). Keep an eye on diabetes. *Nature Clinical Practice Endocrinology and Metabolism, 2*(12).

Filus, A., Trzmiel, A., Kuliczkowska-Płaksej, J., Tworowska, U., Jędrzejuk, D., Milewicz, A., et al. (2008). Relationship between vitamin D receptor BsmI and FokI polymorphisms and anthropometric and biochemical parameters describing metabolic syndrome. *The Aging Male, 11*(3), 134–139.

Gibbs, R. A., Nguyen, P.-N., & Caskey, C. T. (1989). Detection of single DNA base differences by competitive oligonucleotide priming. *Nucleic Acids Research, 17*(7), 2437-2448

Gross, C., Eccleshall, T. R., Malloy, P. J., Villa, M. L., Marcus, R. A., & Feldman, D. L. (1996). The presence of a polymorphism at the translation initiation site of the

vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *Journal of Bone and Mineral Research, 11*(12), 1850-1855.

Hagström, E., Hellman, P., Larsson, T., Ingelsson, E., Berglund, L., Sundström, J., et al. (2009). Plasma parathyroid hormone and the risk of cardiovascular mortality in the community. *Circulation, 119*(21), 2765-2771.

Hellard, S. L., Ballereau, S. J., Visscher, P. M., Torrance, H. S., Pinson, J., Morris, S. W., et al. (2002). SNP genotyping on pooled DNAs: comparison of genotyping technologies and a semi automated method for data storage and analysis. *Nucleic Acids Research, 30*(15), e74-e84.

Hewison, M., Burke, F., Evans, K. N., Lammas, D. A., Sansom, D. M., Liu, P., et al. (2007). Extra-renal 25-hydroxyvitamin D3-1α-hydroxylase in human health and disease. *The Journal of Steroid Biochemistry and Molecular Biology, 103*(3-5), 316-321.

Hewison, M., Gacad, M. A., Lemire, J., & Adams, J. S. (2001). Vitamin D as a cytokine and hematopoetic factor. *Reviews in Endocrine & Metabolic Disorders, 2*(2), 217-227.

Hirai, M., Suzuki, S., Hinokio, Y., Hirai, A., Chiba, M., Akai, H., et al. (2000). Variations in vitamin D-binding protein (group-specific component protein) are associated with fasting plasma insulin levels in Japanese with normal glucose tolerance. *Journal of Clinical Endocrinology and Metabolism, 85*(5), 1951-1953.

Holick, M. F. (2008). The vitamin D deficiency pandemic and consequences for nonskeletal health: Mechanisms of action. *Molecular Aspects of Medicine, 29*(6), 361-368.

Holick, M. F., Biancuzzo, R. M., Chen, T. C., Klein, E. K., Young, A., Bibuld, D., et al. (2008). Vitamin D2 is as effective as vitamin D3 in maintaining circulating concentrations of 25-hydroxyvitamin D. *The Journal of Clinical Endocrinology & Metabolism, 93*(3), 677–681.

Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America, 88*(16), 7276–7280.

Hossein-nezhad, A., Mirzaei, K., Shabani, P., Najmafshar, A., Emamgholipour, S., Rahmani, M., et al. (2009). Association of VDR gene polymorphism with insulin resistance in diabetic patient's running title: VDR and insulin resistance. *Iranian Journal of Diabetes and Lipid Disorders, 8*(1), 143-150.

Human Genome Variation Genotype-to-Phenotype database (HGVbaseG2P). (2009). Retrieved May 21, 2010, from http://www.hgvbaseg2p.org/index

Hurst, P. R. v., Stonehouse, W., & Coad, J. (2010). Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient-a randomised, placebo-controlled trial. *British Journal of Nutrition, 103(4)*, 549-555.

Hurst, P. R. v., Stonehouse, W., Matthys, C., Conlon, C., Kruger, M. C., & Coad, J. (2008). Study protocol - Metabolic syndrome, vitamin D and bone status in South Asian women living in Auckland, New Zealand: A randomised, placebo-controlled, double-blind vitamin D intervention. *BMC Public Health, 8*, 1-8.

Ismail, A., & Namala, R. (2000). Impaired glucose tolerance in vitamin d deficiency can be corrected by calcium *Journal of Nutritional Biochemistry, 11*(3), 170-175

Jablonski, N. G., & Chaplin, G. (2000). The evolution of human skin coloration. *Journal of Human Evolution, 39*(1), 57-106.

Jacobus, C. H., Holick, M. F., Shao, Q. X., Chen, T. C. P., Holm, I. A., Kolodny, J. M., et al. (1992). Hypervitaminosis D associated with drinking milk. *The New England journal of medicine, 326*(18), 1173-1177.

Jafar, T., Tripathi, G., Mehndi, A. A., Mandal, K., Gulati, S., Sharma, R. K., et al. (2009). Vitamin D receptor gene polymorphisms in Indian children with idiopathic nephrotic syndrome. *International Journal of Human Genetics, 9*(1), 49-55.

Jehan, F., d'Alésio, A., & Garabédiana, M. (2007). Exons and functional regions of the human vitamin D receptor gene around and within the main 1a promoter are well conserved among mammals. *The Journal of Steroid Biochemistry and Molecular Biology, 103*(3-5), 361-367.

John, E. M., Schwartz, G. G., Koo, J., Berg, D. V. D., & Ingles, S. A. (2005). Sun exposure, vitamin D receptor gene polymorphisms, and risk of advanced prostate cancer. *Cancer Research, 65*(12), 5470-5474.

Johnson, J. A., Grande, J. P., Roche, P. C., & Kumar, R. (1994). Immunohistochemical localization of' the 1, 25(OH)2D3 receptor and calbindin D28k in human and rat pancreas. *American Journal of Physiology Endocrinology Metabolism, 267*, E356 - E360.

Jurutka, P. W., Remus, L. S., Whitfield, G. K., Thompson, P. D., Hsieh, J.-C., Zitzer, H., et al. (2000). The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB. *Molecular endocrinology, 14*(3), 401-420.

Kállay, E., Bonner, E., Wrba, F., Thakker, R. V., & Peterlik, M. (2002). Molecular and functional characterization of the extracellular calcium-sensing receptor in human colon cancer cells. *Oncology Research, 13*(12), 551-559.

Kerner, S. A., Scott, R. A., & Pike, J. W. (1989). Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3. *Proceedings of the National Academy of Sciences, USA, 86*(12), 4455 - 4459.

Kim, H. S., Newcomb, P. A., Ulrich, C. M., Keener, C. L., Bigler, J., Farin, F. M., et al. (2001). Vitamin D recpetor polymorphism and the risk of colorectal adenomas: Evidence of interaction with dietary vitamin D and calcium. *Cancer Epidemiology, Biomarkers & Prevention, 10*, 869-874.

Kim, S., & Misra, A. (2007). SNP genotyping: Technologies and biomedical applications. *Annual Review of Biomedical Engineering, 9*, 289-320.

Kolb, H., & Mandrup-Poulsen, T. (2005). An immune origin of type 2 diabetes? *Diabetologia, 48*(6), 1038–1050.

Kolek, O. I., Hines, E. R., Jones, M. D., LeSueur, L. K., Lipko, M. A., Kiela, P. R., et al. (2005). 1α, 25-dihydroxyvitamin D3 upregulates FGF23 gene expression in bone: The final link in a renal-gastrointestinal-skeletal axis that controls phosphate transport. *American Journal of Physiology - Gastrointestinal and Liver Physiology, 289*(6 52-6), G1036-G1042.

Kuningas, M., Mooijaart, S. P., Jolles, J., Slagboom, P. E., Westendorp, R. G. J., & Heemst, D. v. (2009). VDR gene variants associate with cognitive function and depressive symptoms in old age. *Neurobiology of Aging, 30*(3), 466-473.

Kwok, P.-Y. (2001). Methods for genotyping single nucleotide polymorphisms. *Annual Review of Genomics and Human Genetics, 2*, 235-258.

Lamberg-Allardt, C. J. E., Outila, T. A., Kärkkäinen, M. U. M., Rita, H. J., & Valsta, L. M. (2001). Vitamin D deficiency and bone health in healthy adults in Finland: Could this be a concern in other parts of Europe? *Journal of Bone and Mineral Research, 16*(11), 2066-2073

Landegren, U., Kaiser, R. J., Sanders, J. Z., & Hood, L. (1988). A ligase-mediated gene detection technique. *Science, 241*(4869), 1077-1080.

Lawson, M., Thomas, M., & Hardiman, A. (1999). Dietary and lifestyle factors affecting plasma vitamin D levels in Asian children living in England. *European Journal of Clinical Nutrition, 53*, 268-272.

Lee, P., Greenfield, J. R., Seibel, M. J., Eisman, J. A., & Center, J. R. (2009). Adequacy of vitamin D replacement in severe deficiency is dependent on body mass index. *The American Journal of Medicine, 122*(11), 1056-1060.

Lehmann, B. (2009). Role of the vitamin D3 pathway in healthy and diseased skin - Facts, contradictions and hypotheses. *Experimental Dermatology, 18*(2), 97-108.

Levy, J. C., Matthews, D. R., & Hermans, M. P. (1998). Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care, 21*(12), 1291-1293.

Li, H., Stampfer, M. J., Hollis, J. B. W., Mucci, L. A., Gaziano, J. M., Hunter, D., et al. (2007). A prospective study of plasma vitamin D metabolites, vitamin D receptor polymorphisms, and prostate cancer. *PloS Medicine, 4*(3), 562-571.

Li, Y. t., Kong, J., Wei, M., Chen, Z. F., Liu, S. Q., & Cao, L. P. (2002). 1, 25-Dihydroxyvitamin D3 is a negative endocrine regulator of the renin-angiotensin system. *Journal of Clinical Investigation, 110*(2), 229-238

Lins, T. C. L., Nogueira, L. R., Lima, R. M., Gentil, P., Oliveira, R. J., & Pereira, R. W. (2007). A multiplex single-base extension protocol for genotyping Cdx2, FokI, BsmI, ApaI, and TaqI polymorphisms of the vitamin D receptor gene. *Genetics and Molecular Research, 6*(2), 316-324.

Livak, K. J. (1999). Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genetic Analysis - Biomolecular Engineering, 14*(5-6), 143-149.

Macdonald, H. M., McGuigan, F. E., Stewart, A., Black, A. J., Fraser, W. D., Ralston, S., et al. (2006). Large-scale population-based study shows no evidence of association between common polymorphism of the VDR gene and BMD in British women. *Journal of Bone and Mineral Research, 21*(1), 151-162.

Malecki, M. T., Klupa, T., Wanic, K., Cyganek, K., Frey, J., & Sieradzki, J. (2002). Vitamin D binding protein gene and genetic susceptibility to type 2 diabetes mellitus in a Polish population. *Diabetes Research and Clinical Practice, 57*(2), 99-104.

Markestad, T., Hesse, V., Siebenhuner, M., Jahreis, G., Aksnes, L., Plenert, W., et al. (1987). Intermittent high-dose vitamin D prophylaxis during infancy: effect on vitamin D metabolites, calcium, and phosphorus. *American Journal of Clinical Nutrition, 46*(4), 652-658.

Martin, D. L., & Deluca, H. F. (1969). Calcium transport and the role of vitamin D. *Archives of Biochemistry and Biophysics, 134*(1), 139-148.

Martin, K. J., & González, E. A. (2004). Vitamin D analogs: Actions and role in the treatment of secondary hyperparathyroidism. *Seminars in Nephrology, 24*(5), 456-459.

McCullough, M. L., Bostick, R. M., & Mayo, T. L. (2009). Vitamin D gene pathway polymorphisms and risk of colorectal, breast, and prostate cancer. *Annual Review of Nutrition, 29*, 111-132.

McDermott, M. F., Ramachandran, A., Ogunkolade, B. W., Aganna, E., Curtis, D., & Boucher, B. J. (1997). Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians. *Diabetologia, 40*, 971-975.

McDonnell, D. P., Scott, R. A., Kerner, S. A., O'Malley, B. W., & Pike, J. W. (1989). Functional domains of the human vitamin D3 receptor regulate osteocalcin gene expression. *Molecular Endocrinology, 3*(4), 635-644.

McKenna, N. J., Lanz, R. B., & O'Malley, B. W. (1999). Nuclear receptor coregulators: Cellular and molecular biology. *Endocrine Reviews, 20*(3), 321-344.

Michalatos-Beloin, S., Tishkoff, S. A., Bentley, K. L., Kidd, K. K., & Ruano, G. (1996). Molecular haplotyping of genetic markers 10 kb apart by allele-specific long-range PCR. *Nucleic Acids Research, 24*(23), 4841-4843.

Ministry of Health. (2006). *Asian Health Chart Book 2006 Public Health Intelligence* (No. 4). Wellington: Ministry of Health. Retrieved from http://www.moh.govt.nz/moh.nsf/pagesmh/4925/$File/asian-health-chart-book-2006.pdf

Mitra, S., Desai, M., & Khatkhatay, M. I. (2006). Vitamin D receptor gene polymorphisms and bone mineral density in postmenopausal Indian. *Maturitas, 55*(1), 27-35.

Mittal, R. D., Manchanda, P. K., Bhat, S., & Bid, H. K. (2007). Association of vitamin-D receptor (Fok-I) gene polymorphism with bladder cancer in an Indian population. *BJU International, 99*(4), 933-937

Miyamoto, K.-i., Kesterson, R. A., Yamamoto, H., Taketani, Y., Nishiwaki, E., Tatsumi, S., et al. (1997). Structural Organization of the Human Vitamin D Receptor Chromosomal Gene and Its Promoter. *Molecular Endocrinology, 11*(8), 1165-1179.

Morrison, N. A., Qi, J. C., Tokita, A., Kelly, P. J., Crofts, L., Nguyen, T. V., et al. (1994). Prediction of bone density from vitamin D receptor alleles. *Nature, 367*(6460), 284-287.

Muniyappa, R., Montagnani, M., Koh, K. K., & Quon, M. J. (2007). Cardiovascular actions of insulin. *Endocrine Reviews, 28*(5), 463 - 491.

New Zealand Guidelines Group. (2003). *Management of type 2 diabetes*. Retrieved 12 May, 2010, from http://www.nzgg.org.nz/guidelines/0036/Diabetes_summary.pdf

Nishimura, A., Shinki, T., Cheng, H. J., Ohyama, Y. I., Noshiro, M., Okuda, K. I., et al. (1994). Regulation of messenger ribonucleic acid expression of 1α, 25-

dihydroxyvitamin D3-24-hydroxylase in rat osteoblasts. *Endocrinology, 134*(4), 1794-1799

Norman, A. W. (2006). Minireview: Vitamin D receptor: New assignments for an already busy receptor. *Endocrinology, 147*(12), 5542-5548.

Ntais, C., Polycarpou, A., & Ioannidis, J. P. A. (2003). Vitamin D Receptor Gene Polymorphisms and Risk of Prostate Cancer: A Meta-analysis. *Cancer Epidemiology, Biomarkers & Prevention, 12*, 1395–1402.

Ochs-Balcom, H. M., Cicek, M. S., Thompson, C. L., Tucker, T. C., Elston, R. C., Plummer, S. J., et al. (2008). Association of vitamin D receptor gene variants, adiposity and colon cancer. *Carcinogenesis, 29*(9), 1788–1793.

Ogunkolade, B. W., Boucher, B. J., Prahl, J. M., Bustin, S. A., Burrin, J. M., Noonan, K., et al. (2002). 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, 51*(7), 2294-2300.

Oh, J.-Y., & Barrett-Connor, E. (2002). Association between vitamin D receptor polymorphism and type 2 diabetes or metabolic syndrome in community-dwelling older adults: The Rancho Bernardo Study. *Metabolism, 51*(3), 356-359.

Okayama, N., Fujimura, K., Nakamura, J., Suehiro, Y., Hamanaka, Y., & Hinoda, Y. (2004). Evaluation of a new efficient procedure for single-nucleotide polymorphism genotyping: tetra-primer amplification refractory mutation system-polymerase chain reaction. *Clinical Chemistry and Laboratory Medicine, 42*(1), 13-16

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

Patil, N., Berno, A. J., Hinds, D. A., Barrett, W. A., Doshi, J. M., Hacker, C. R., et al. (2001). Blocks of limited haplotype diversity revealed by high-resolution scanning of human chromosome 21. *Science, 294*(5547), 1719-1723.

Plum, L. A., & DeLuca, H. F. (2009). The functional metabolism and molecular biology of vitamin D action. *Clinical Reviews in Bone and Mineral Metabolism, 7*, 20-41.

Quesada, J. M., Casado, A., Díaz, C., Barrios, L., Cuenca-Acevedo, R., & Doradoc, G. (2004). Allele-frequency determination of BsmI and FokI polymorphisms of the VDR gene by quantitative real-time PCR (QRT-PCR) in pooled genomic DNA samples. *The Journal of Steroid Biochemistry and Molecular Biology, 89-90*, 209-214.

Ragoussis, J. (2007). Genotyping technologies for genetic research. *Annual Review of Genomics and Human Genetics, 10*, 117-133.

Reaven, G. (2005). Insulin resistance, type 2 diabetes mellitus, and cardiovascular disease: The end of the beginning. *Circulation, 112*(20), 3030-3032.

Reis, A. F., Hauache, O. M., & Velho, G. (2005). Vitamin D endocrine system and the genetic susceptibility to diabetes, obesity and vascular disease. A review of evidence. *Diabetes and Metabolism, 31*(4i), 318-325.

Rezende, V. B., Jr., F. B., Montenegro, M. F., Sandrim, V. C., Gerlach, R. F., & Tanus-Santos, J. E. (2007). An interethnic comparison of the distribution of vitamin D receptor genotypes and haplotypes. *Clinica Chimica Acta, 384*(1-2), 155-159.

Richards, M. L., & Thompson, N. W. (1999). Diabetes mellitus with hyperparathyroidism: Another indication for parathyroidectomy? *Surgery, 126*(6), 1160-1166

Ross, P., Hall, L., Smirnov, I., & Haff, L. (1998). High level multiplex genotyping by MALDI-TOF mass spectrometry. *Nature Biotechnology, 16*(13), 1347-1351

Rukin, N. J., & Strange, R. C. (2007). What are the frequency, distribution, and functional effects of vitamin D receptor polymorphisms as related to cancer risk? *Nutrition Reviews, 65*(8), S96-S101.

Scragg, R., Holdaway, I., Singh, V., Metcalf, P., Baker, J., & Dryson, E. (1995). Serum 25-hydroxyvitamin D3 levels decreased in impaired glucose tolerance and diabetes mellitus. *Diabetes Research and Clinical Practice, 27*(3), 181-188.

Senn, J. J., Klover, P. J., Nowak, I. A., Zimmers, T. A., Koniaris, L. G., Furlanetto, R. W., et al. (2003). Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *Journal of Biological Chemistry, 278*(16), 13740-13746.

Sergeev, I. N., & Rhoten, W. B. (1995). 1, 25-Dihydroxyvitamin D3 evokes oscillations of intracellular calcium in a pancreatic β-cell line. *Endocrinology 136*(7), 2852-2861.

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Yoneya, T., et al. (2004). FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *Journal of Bone and Mineral Research, 19*(3), 429-435

Shimada, T., Kakitani, M., Yamazaki, Y., Hasegawa, H., Takeuchi, Y., Fujita, T., et al. (2004). Targeted ablation of FGF23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *Journal of Clinical Investigation, 113*(4), 561-568.

Single nucleotide polymorphism. Retrieved November 15, 2009, from http://www.ncbi.nlm.nih.gov/projects/SNP/

Slattery, M. L. (2007). Vitamin D receptor gene (VDR) associations with cancer. *Nutrition Reviews, 65*(8), S102-S104.

Slattery, M. L., Yakumo, K., Hoffman, M., & Neuhausen, S. (2001). Variants of the VDR gene and risk of colon cancer. *Cancer Causes and Control, 12*(4), 359-364.

Smith, K. (2002). Genetic polymorphism and SNPs: Genotyping, haplotype assembly problem, haplotype map, functional genomics and proteomics. 1-13. Retrieved from http://www.cs.mcgill.ca/~kaleigh/compbio/snp/snp_summary.pdf

Sokolov, B. P. (1989). Primer extension technique for the detection of single nucleotide in genomic DNA. *Nucleic Acids Research, 18*(12), 3671.

Song, Y., & Manson, J. E. (2010). Vitamin D, insulin resistance, and type 2 diabetes. *Current Cardiovascular Risk Reports, 4*, 40-47.

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

Tanaka, Y., Seino, Y., & Ishida, M. (1986). Effect of 1, 25-dihydroxyvitamin D3 on insulin secretion: Direct or mediated? *Endocrinology, 118*(5), 1971-1976.

Taylor, A. V. G., & Wise, P. H. (1998). Vitamin D replacement in Asians with diabetes may increase insulin resistance. *Postgraduate Medical Journal, 74* (872), 365-366.

Taylor, S. I., Accili, D., Cama, A., Imano, E., Kadowaki, H., & Kadowaki, T. (1991). Unusual forms of insulin resistance. *Annual Review of Medicine, 42*, 373-379.

Thakkinstian, A., D'Este, C., & Attia, J. (2004). Haplotype analysis of VDR gene polymorphisms: A meta-analysis. *Osteoporosis International, 15*(9), 729-734.

Thakkinstian, A., D'Este, C., Eisman, J., Nguyen, T., & Attia, J. (2004). Meta-analysis of molecular association studies: Vitamin D receptor gene polymorphisms and BMD as a case study. *Journal of Bone and Mineral Research, 19*(3), 419-428.

Tong, A. K., Li, Z., Jones, G. S., Russo, J. J., & Ju, J. (2001). Combinatorial fluorescence energy transfer tags for multiplex biological assays. *Nature Biotechnology, 19*(8), 756-759.

Turunen, M. M., Dunlop, T. W., Carlberg, C., & Vaisanen, S. (2007). Selective use of multiple vitamin D response elements underlies the 1 α, 25-dihydroxyvitamin D3-mediated negative regulation of the human CYP27B1 gene. *Nucleic Acids Research, 35*(8), 2734-2747.

Tworowska-Bardzínska, U., Lwow, F., Kubicka, E., Łaczmanski, Ł., Jędzrzejuk, D., Dunajska, K., et al. (2008). The vitamin D receptor gene BsmI polymorphism is not associated with anthropometric and biochemical parameters describing metabolic syndrome in postmenopausal women. *Gynecological Endocrinology, 24* (9), 514-518.

Uitterlinden, A. G., Fang, Y., Bergink, A. P., Meurs, J. B. J. v., Leeuwen, H. P. T. M. v., & Pols, H. A. P. (2002). The role of vitamin D receptor gene polymorphisms in bone biology. *Molecular and Cellular Endocrinology, 197*(1-2), 15-21.

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

Uitterlinden, A. G., Pols, H. A. P., Burger, H., Huang, Q., Daele, P. L. A. V., & Duijn, C. M. V. (1996). A large-scale population-based study of the association of vitamin D receptor gene polymorphisms with bone mineral density. Journal *of Bone and Mineral Research, 11*(9), 1241-1248.

Uitterlinden, A. G., Ralston, S. H., Brandi, M. L., Carey, A. H., Grinberg, D., Langdahl, B. L., et al. (2006). The association between common vitamin D receptor gene variations and osteoporosis: A participant-level meta-analysis. *Annals of Internal Medicine, 145*(4), 255-264.

Utiger, R. D. (1998). The need for more vitamin D. *The New England Journal of Medicine, 338* (12), 828-829.

Veldman, C. M., Cantorna, M. T., & DeLuca, H. F. (2000). Expression of 1, 25-dihydroxyvitamin D3 receptor in the immune system. *Archives of Biochemistry and Biophysics, 374*(2), 334–338.

Vupputuri, M. R., Goswami, R., Gupta, N., Ray, D., Tandon, N., & Kumar, N. (2006). Prevalence and functional significance of 25-hydroxyvitamin D deficiency and vitamin D receptor gene polymorphisms in Asian Indians. *The American Journal of Clinical Nutrition, 83*(6), 1411-1419.

Wallace, T. M., Levy, J. C., & Matthews, D. R. (2004). Use and abuse of HOMA modeling. *Diabetes Care, 27*(6), 1487-1496.

Walters, M. R. (1992). Newly identified actions of the vitamin D endocrine system. *The Endrocrine Society, 13*(4), 719-764.

Whitfield, G. K., Remus, L. S., Jurutka, P. W., Zitzer, H., Oza, A. K., Dang, H. T. L., et al. (2001). Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. *Molecular and Cellular Endocrinology, 177*(1-2), 145-159.

Wielen, R. P. J. v. d., Groot, L. C. P. G. M. d., Staveren, W. A. v., Löwik, M. R. H., Berg, H. v. d., Haller, J., et al. (1995). Serum vitamin D concentrations among elderly people in Europe. *The Lancet, 346*(8969), 207-210

Wong, R. G., Norman, A. W., Reddy, C. R., & Coburn, J. W. (1972). Biologic effects of 1, 25-dihydroxycholecalciferol (a highly active vitamin D metabolite) in acutely uremic rats. *The Journal of Clinical Investigation Volume, 51*(5), 1287-1291

Xiang, W., Kong, J., Chen, S., Cao, L.-P., Qiao, G., Zheng, W., et al. (2005). Cardiac hypertrophy in vitamin D receptor knockout mice: Role of the systemic and cardiac renin-angiotensin systems. *American Journal of Physiology Endocrinology and Metabolism, 288*, E125–E132.

Zierold, C., Darwish, H. M., & DeLuca, H. F. (1995). Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *The Journal of Biological Chemistry, 270*(4), 1675-1678.

Zittermann, A. (2003). Vitamin D in preventive medicine: Are we ignoring the evidence? *British Journal of Nutrition, 89*, 552-572.

Appendices

Genotype and haplotype data for 239 South-Asian women

Subject #	*Cdx-2*	*FokI*	*BsmI*	*ApaI*	*TaqI*	*3' haplotype*
1	GG	Ff	bb	aa	TT	baT,baT
2	GA	FF	BB	AA	tt	BAt,BAt
3	GA	ff	BB	AA	tt	BAt,BAt
6	GA	Ff	Bb	AA	tt	BAt,bAt
7	GA	FF	BB	AA	tt	BAt,BAt
8	GA	FF	bb	aa	TT	baT,baT
9	GG	FF	Bb	Aa	Tt	baT,BAt
10	GA	Ff	bb	aa	TT	baT,baT
11	GG	FF	bb	aa	TT	baT,baT
12	GA	Ff	bb	aa	TT	baT,baT
14	GA	FF	bb	aa	TT	baT,baT
15	GG	ff	Bb	Aa	Tt	BAt,baT
16	GA	FF	bb	Aa	TT	bAT,baT
17	GA	FF	bb	AA	TT	bAT,bAT
18	GG	Ff	bb	aa	TT	baT,baT
19	GG	Ff	bb	Aa	TT	bAT,baT
20	GA	FF	Bb	Aa	Tt	BAt,baT
21	GG	Ff	Bb	Aa	Tt	BAt,baT
22	GA	FF	Bb	Aa	TT	baT,BAT
23	GG	Ff	Bb	Aa	TT	baT,BAT
24	GG	Ff	Bb	Aa	Tt	baT,BAt
25	GG	FF	Bb	Aa	Tt	BAt,baT
26	GG	FF	BB	AA	tt	BAt,BAt
27	GG	FF	Bb	Aa	Tt	BAt,baT
28	GG	Ff	Bb	AA	TT	BAT,bAT
29	AA	FF	bb	Aa	TT	bAT,baT
30	GA	FF	Bb	Aa	TT	BAT,baT
32	GG	ff	Bb	Aa	Tt	BAt,baT
33	GG	Ff	BB	AA	Tt	BAT,BAt
34	GG	FF	BB	AA	Tt	BAT,BAt
35	GA	FF	Bb	Aa	TT	BAT,baT
36	GA	Ff	Bb	AA	Tt	BAt,bAT
37	GA	Ff	Bb	Aa	Tt	BAt,baT
38	GG	Ff	Bb	Aa	Tt	BAt,baT
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43	AG	FF	Bb	Aa	TT	BAT,baT
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45	AA	FF	Bb	Aa	Tt	BAt,baT
46	GG	Ff	bb	aa	TT	baT,baT
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51	AG	Ff	bb	Aa	TT	bAT,baT
52	AG	FF	bb	aa	TT	baT,baT
53	AG	FF	bb	Aa	TT	bAT,baT
54	GG	Ff	bb	aa	TT	baT,baT

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277	AG	FF	BB	AA	Tt	BAT,BAt
278	GG	FF	bb	Aa	TT	bAT,baT
279	AG	FF	Bb	Aa	TT	BAT,baT

List of abbreviations

AS-PCR	Allele-specific polymerase chain reaction
ASM-PCR	Allele specific multiplex polymerase chain reaction
BMD	Bone mineral density
BMI	Body mass index
CaSR	Calcium receptors
COL2A1	Collagen type II alpha I
CPE	Common primer extension
CV	Cardiovascular diseases
CUPS-7	Chennai Urban Population Study-7
DBP	Vitamin D binding protein
ddNTP	Dideoxy nucleotide triphosphate
FGF 23	Fibroblast growth factor 23
FSG	Fasting serum glucose
FSI	Fasting serum insulin
gDNA	Genomic DNA
7-HDC	7-dehydrocholesterol
HOMA	Homeostasis assessment model 1
HOMA-IR	Homeostasis assessment model 1 for insulin resistance
HOMA%S	Homeostasis assessment model 1 for insulin sensitivity
HOMA2	Homeostasis assessment model 2
HOMA2%S	Homeostasis assessment model 2 for insulin sensitivity
HDL-C	High density lipoprotein cholesterol
HW	Hardy Weinberg equilibrium
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
LD	Linkage disequilibrium
LDL-C	Low density lipoprotein cholesterol

MS	Multiple sclerosis
NTC	No template control
OA	Osteoarthritis
PTH	Parathyroid hormone
PCR	Polymerase chain reaction
qPCR	Quantitative/Real-time polymerase chain reaction
RXR	Retinoid acid X receptor
RFLP-PCR	Restriction fragment length polymorphism polymerase chain reaction
SNP	Single nucleotide polymophism
SPE	Specific primer extension
SD	Standard deviation
TNF	Tumor necrosis factor
TBE	Tris borate EDTA
TAE	Tris acetate EDTA
TG	Triglyceride
TC	Total cholesterol
UVR	Ultraviolet radiation
VDR	Vitamin D receptor
VDRE	Vitamin D response elements
VNTR	Variable number of tandem repeats

Ethical approval

Massey University

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20 December 2006

Ms Pamela von Hurst
IFNHH
ALBANY

Dear Pamela

Re: HEC: Southern A Application – 06/67
An investigation of the relationship between nutrition and risk factors for cardiovascular disease, diabetes and osteoporosis in South Asian Indian women living in New Zealand with follow-up vitamin D intervention study

Thank you for your letter dated 20 December 2006.

On behalf of the Massey University Human Ethics Committee: Southern A, I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

J. O'Neill

Professor John O'Neill, Chair
Massey University Human Ethics Committee: Southern A

cc Dr Jane Coad
IFNHH
PN452

Prof Richard Archer, HoI
IFNHH
PN452

Institute of Food, Nutrition and Human Health
Massey University
Albany, Auckland, New Zealand

Surya Study
An investigation of the relationship between nutrition and risk factors for disease in South Asian women

Information for Participants

You are invited to take part in a university research project investigating the nutritional status of South Asian women, and its relationship with risk factors for diabetes, cardiovascular disease and bone health. The principal investigator (details below) is a PhD candidate in Nutritional Science at Massey University.

Principal Investigator:	**Supervisor:**
Pamela von Hurst	Dr Jane Coad
Institute of Food, Nutrition and Human Health	Institute of Food, Nutrition and Human Health
Massey University, Albany	Massey University, Palmerston North
Tel: 414 0800 ext 41205	Tel: 414 0800 ext 5962
Email: P.R.vonHurst@massey.ac.nz	Email: J.Coad@massey.ac.nz

We are looking for 300-350 South Asian women to participate in this study.
To fit in to our study you should:

Have been born in South Asia (or have parents or grandparents who were born there).
Be female, over 20 years of age.
Be willing to fast overnight for 10 to 12 hours and not drink any alcohol on the day before you come into the lab.
Not be pregnant, breast-feeding or planning in the near future.
Not be taking any medications which might interfere with our tests (we will ask you questions about your health).

If you decide you would like to take part in this study you will be first asked to complete a questionnaire which includes a medical history to ensure that you fit the needs of the study. If you do, we will then invite you to the Human Nutrition Unit at Massey University or another Auckland centre, where you will spend an hour with a researcher who will measure and weigh you, and take your blood pressure. You will be asked to provide a blood sample of about 25ml (about 5 tea-spoons) which will be taken at the same time. You will be asked to complete another questionnaire about your beliefs and knowledge about osteoporosis and a medical history form. Finally, you will be given a diary to

complete with details about the foods you eat and what activities (such as walking and exercise) you do during the period of a week.

About the study

Phase 1 of the study seeks to recruit 300 women who were themselves born in the South Asian continent, or whose parents or grandparents were born there. The study is intended to find out about the nutritional status of South Asian women, how much vitamin D they get from their normal diet and daily activities, and how their diet affects other aspects of their health especially glucose tolerance and bone health.
Involvement in this part of the study would include the following:

- The researcher would make an appointment for you to visit the Massey University, Albany campus or another Auckland location, early in the morning before you have breakfast. We will reimburse you for your travel costs. You would first have blood samples taken (about 25 ml which is equivalent to about 5 tea-spoons). This "Fasting blood sample" needs to be taken early in the morning before you eat or drink (other than water). You also need to avoid alcohol in the 24-hour period before the blood test.

- Some of the blood taken will be analysed for calcium, vitamin D, glucose and insulin levels, B vitamins, lipid and cholesterol levels, fibrinogen and parathyroid hormone (which affects calcium handling by the body). Part of the sample will be stored at the laboratory at Massey University where a genetic analysis will be done to look for variations in the vitamin D receptor gene when the blood samples from all the subjects in the study have been collected.

- Next you would be offered a light breakfast meal (of fruit, cereal, milk, yoghurt, bread, tea and coffee). We will then measure your height, weight and waist and hip circumference. All measurements will be made by female researchers over light clothing so you do not need to get undressed. We will then also take your blood pressure measurement

- The researcher will give you a food and activity diary for you to complete over the following week or so and mail back.

We would also like to invite you to return to Massey at a later date to give you a bone density scan. DEXA bone densitometry is quick, accurate, non-invasive method of assessing bone health and risk for osteoporosis (fragile bones in older age) which does not involve anaesthetic. The amount of X-ray dose is very small (about the same as the average person receives from background radiation in one day or less than one tenth of the dose used in a standard chest X-ray).

It is possible that results from the blood tests in phase 1 of the study might show some potentially abnormal results (such as very high blood glucose levels or very low vitamin D levels) that should be investigated further. If any such problems are identified, we will invite you to come back to Massey to discuss these with the study doctor, Dr Shashikala Bhuthoji, who is a registered medical practitioner who works in the Massey University Health Centre. She may advise you to contact your General Practitioner or will contact your General Practitioner on your behalf if you prefer.

Phase 2

Phase 2 of the study seeks to recruit 100 women from the first part of the study who were found to have high blood glucose levels or other markers of metabolic syndrome in the blood sample taken at the start of the study but are not taking any medication for diabetes. This part of the study will investigate the relationship between vitamin D and glucose intolerance. It is an intervention study which means that participants will be given either 4 small capsules containing a vitamin D supplement, or 4 small capsules of identical appearance which do not contain vitamin D, daily for a period of 6 months. During that period we will assess your vitamin status and blood glucose and insulin levels.

Participation in the study would also involve visiting a Diagnostic MedLab on 3 other occasions to have a fasting blood sample taken (in total about 19 mls which is equivalent to about 4 tea-spoons). These need to be taken early in the morning before you eat.

At some point during the 6 month period, you will need to come to Massey University Nutrition Laboratory where we will give you a bone density scan. DEXA bone densitometry is quick, accurate, non-invasive method of assessing bone health and risk for osteoporosis (fragile bones in older age) which does not involve anaesthetic. The amount of X-ray dose is very small (about the same as the average person receives from background radiation in one day or less than one tenth of the dose used in a standard chest X-ray).

Risks and benefits

There will be no charges made for any of the tests that you undertake. The principal benefit of taking part in this study is that you contribute to our better understanding of the health problems of South Asians in New Zealand. Over recent years the number people from these regions living in New Zealand has greatly increased but we know very little about the health of this growing segment of our population.

There are no personal risks to your health, but the blood tests and bone density scan could potentially identify undiagnosed health problems. If any such problems are identified, we will invite you to come back to Massey to discuss these with the study doctor, Dr Shashikala Bhuthoji, who is a registered medical practitioner who works in the Massey University Health Centre. She may advise you to contact your General Practitioner. At your request, we would be happy to contact your General Practitioner for you.

Participation

You are under no obligation to accept this invitation to take part in this research study. If you decide to participate, you have the right to:

- decline to answer any particular question;
- withdraw from the study (at any time without having to give a reason);
- ask any questions about the study at any time during participation;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be given access to a summary of the project findings when it is concluded

General

If you want to discuss any aspect of this study you should contact the Principal Investigator, Pamela von Hurst at Massey University, 414 0800 ext. 41205.

If you have any queries or concerns regarding your rights as a participant in this study you may wish to contact a Health and Disability Advocate, telephone 0800 555 050.

At the conclusion of the study we will provide a report of the outcome to those involved in the study, we will also hold a meeting to discuss the results which you can attend if you wish. We anticipate that the anonymous results will be published in an international medical journal.

Confidentiality

No material which could personally identify you would be used in any reports on this study. Information collected from you in the study will be stored securely in the Department of Nutrition and will only be available to study personnel, unless you request that we release it some other individual (such as your General Practitioner). When the study is completed, all material will be destroyed.

Genetic testing

Each person has a DNA make-up (their genes) which is different from that of everybody else - except in the case of identical twins. This genetic make-up is a mixture of the genes of our parents. The precise way they are mixed varies from child to child within the same family, so having the same parents does not mean that two children will have exactly the same genes. We already know that some health conditions and disorders are definitely inherited through the genes (hereditary conditions), but we do not know how many conditions are explained by genetic inheritance. Inherited genes may explain why some people are more resistant and some people more prone to disorders which have not yet been identified as hereditary. The research in which you are invited to participate will investigate genetic make-up to look for any link.

Because the research investigates genetic make-up, this identifies a participant and their particular genetic characteristics. This information is confidential and will not be disclosed, stored, or used in any way without the informed consent of the participant.

We have no intention of claiming the right, ownership or property in your individual genetic information or that of your kinship group. You consenting to participate in DNA sampling of the proposed study will not be construed as creating any right or claim on the part of the researcher to your genetic information.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Injury Prevention, Rehabilitation and Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Please feel free to contact the researcher if you have any questions about this study.

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 06/67. If you have any concerns about the conduct of this research, please contact Professor John O'Neill, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 8635, email humanethicssoutha@massey.ac.nz

Conference presentations

Jain, R., Hurst, P. R. v., Coad, J., Love, D. R., & Higgins, C. M. (2010). Are Vitamin D Supplements effective for everyone? Analysis of the vitamin D receptor gene for pharmacogenetic strategies. Poster presentation at the NZBIO 2010 Conference, Auckland

Jain, R., Hurst, P. R. v., Coad, J., Love, D. R., & Higgins, C. M. (2009). When too much sun is never enough: Association of *VDR* gene polymorphisms with hypovitaminosis D. Poster presentation at the Queenstown Molecular Biology Meeting, Queenstown.

Jain, R., Hurst, P. R. v., Coad, J., Love, D. R., & Higgins, C. M. (2009). When too much sun is never enough: Association of *VDR* gene polymorphisms with hypovitaminosis D. Oral presentation at the University Post graduate Symposium, New Direactions, Challenges and Applications at the Auckland University of Technology (AUT), Auckland.