ASSOCIATION OF 25-HYDROXY VITAMIN D LEVELS WITH VITAMIN D RECEPTOR TAQ1 GENE POLYMORPHISM IN CHENNAI POPULATION- A CROSS SECTIONAL STUDY

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CERTIFICATE

This to certify that the dissertation entitled **"ASSOCIATION OF 25-HYDROXY VITAMIN D LEVELS WITH VITAMIN D RECEPTOR TAQ1 GENE POLYMORPHISM IN CHENNAI POPULATION " – A CROSS SECTIONAL STUDY** is the bonafide original work done by **DR.G.POONGODI**, Post graduate in **Biochemistry under** overall supervision and guidance in the Department of Biochemistry, Kilpauk Medical College, Chennai in partial fulfillment of the regulations of The Tamilnadu Dr. M.G.R . Medical University for the award of M.D.Degree in Biochemistry**(Branch XIII).**

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This dissertation is submitted to **THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY** Chennai, in partial fulfillment of the university regulations for the award of **DEGREE OF M.D BIOCHEMISTRY (BRANCH - XIII)** examinations to be held in **APRIL – 2016.**

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CONTENTS

ABSTRACT

TITLE:

"Association of 25-hydroxy vitamin D levels with vitamin D receptor Taq1 gene polymorphism in Chennai population." A cross sectional study.

Vitamin D stands at the frontline of current scientific endeavours, being a topic of great interest to medical researchers all over the globe. Vitamin D deficiency (VDD) has been arising as a pandemic around the world. Globally about one billion people are known to have VDD and it is the most common undiagnosed medical condition

Vitamin D receptor (VDR) gene is located in the chromosome12cen-q12. Taq1 gene is present in the 3'UTR (untranslated region), is a T/C nucleotide substitution (ATT/ATC) in exon IX coding for isoleucine. This could potentially influence the activity of 1, 25 dihydroxy vitamin D [1, 25$(OH)_2$ D] by mRNA instability or by affecting the regulation of translation.

AIM OF THE STUDY:

- To determine the frequency of VDR Taq1 gene polymorphism in normal individuals.
- To correlate the frequency of VDR Taq1 gene polymorphism with 25-hydroxy vitamin D [25(OH)D]levels

- To correlate 25-(OH)D with the levels of parathyroid hormone(PTH), Calcium (Total and Ionized) and Phosphorus

MATERIALS AND METHODS:

The study population consists of 100 normal healthy individuals. Fasting venous blood was collected from each subjects and estimation of 25- hydroxy vitamin D, Intact PTH assay, total and ionized calcium, phosphorus, albumin, total protein, glucose, urea and creatinine were done. VDR Taq1gene Polymorphism was examined using PCR based restriction fragment analysis.

RESULTS:

Out of 100 samples analysed, the genotype frequency of Taq1 **tt** accounts for 94%, **Tt accounts for** 6% and **TT accounts for** 0% respectively. The genotype distribution was not found to be in agreement with Hardy-Weinberg Equilibrium. When genotypes are analysed with respect to 25(OH) D, no correlation was observed with respect to Taq1 SNP.

About 21% having vitamin D deficiency and the remaining were within normal range. The vitamin D level decreases with increase in parathyroid hormone which is statistically significant with p value of 0.002. The total calcium is decreased in VDD subjects.

CONCLUSION:

From this study we conclude that the predominant VDR Taq1genotype is homozygous tt genotype and the homozygous TT genotype is not found in this study population. The prevalence of vitamin D deficiency is about 21%. Also the genotypes does not correlate with vitamin D levels.

Key words:

VDR, Polymorphism, 25-hydroxy vitamin D, Parathyroid hormone, Genotype, Taq1.

INTRODUCTION

To the medical researchers all over the globe vitamin D had become topic of interest. Today the world is facing the problem of vitamin D deficiency pandemic as evidenced by recent research.

Deficiency of vitamin D is a risk factor in humans, right from conception and throughout lifespan[1]. It is one of the common medical condition undiagnosed and under treated nutritional deficiency in the world. Globally about one billion people are known to have vitamin D insufficiency or deficiency[2].

Prevalence of vitamin D deficiency is about 70-100% in general population worldwide. In India the increased prevalence of about 50-100% is due to low intake of dietary calcium[3, 4]. In various groups like school children, adolescents, pregnant women and health care professionals, vitamin D deficiency is highly prevalent.

In late 20th century vitamin D is described as conditional vitamin. Traditionally it is called as sunshine vitamin. India is a tropical country located between 8.4°N and 37.6°N latitude and people living here receive ample sunlight throughout the year despite this hypovitaminosis D is common in India[3,4].

Vitamin D is unique, because the nutrient requirement is met by both endogenous production from sunlight and exogenously by diet in our body.

The epidemic of vitamin D deficiency contribute burden to the health system in India because of its various implications in health.

Vitamin D deficiency (VDD) is noticed in both breast fed infants and older children. It is the common cause of rickets universally[4]. It is called as anti -rachitic factor.

The medical fraternities across the world are curious in realizing that vitamin D plays a major role in health and disease. It causes skeletal as well as extra skeletal manifestations. Vitamin D not only regulates calcium and phosphorus homeostasis but also protects the individuals from many diseases like malignancies, chronic infections, and cardiac problems and reduce the risk of autoimmune diseases.

The study is to impress upon the physicians about the gravity of the vitamin D deficiency problem throughout India and to make appropriate diagnosis and treatment with care and caution[5]. As the vitamin D status is improved worldwide it would have remarkable effects on public health, and decrease the healthcare expenses for several chronic diseases.

Vitamin D exerts its action in the body by binding to vitamin D receptor(VDR) which is a member of nuclear hormone receptor super family.It modulates the transcription of target genes by complexing with vitamin D responsive elements (VDRES) in the promoter region of target genes[6].

Excellent opportunities are provided by genetic studies to relate molecular insights with epidemiological data and it gained much interest. Polymorphism referred as DNA sequence variations which occur frequently in the population and can have only modest and subtle effects.

Many polymorphisms are known to exist in VDR gene according to recent studies, but the influence of this polymorphism on VDR protein function is not known. At the 3'end of the VDR gene three restriction fragments length polymorphisms (RFLP) for BsmI, ApaI and TaqI have been studied so far.

VDR gene polymorphisms could potentially influence the binding of 1, 25(OH) $_2$ D and the anti-proliferative effects of vitamin. Many studies have been reported on VDR polymorphism and its influence on different disease throughout the world. On the contrary in our country the reports are minimal.

A T/C nucleotide substitution (ATT to ATC) leading to a silent change at codon 352 (isoleucine) in exon IX is the Taq1 polymorphism[7].

Therefore this study is to find the frequency of VDR (Taq1) gene polymorphism by PCR based restriction analysis of normal individuals in Chennai and to determine the association of this polymorphism with 25-hydroxy vitamin D levels.

REVIEW OF LITERATURE

Gold blatt and Soames discovered that vitamin D is produced by irradiation of 7-dehydrocholesterol in skin[9]. The structure of vitamin D_2 and D_3 was described by Windaus, German chemist after irradiation of ergosterol or 7-dehydrosterol. He had been awarded the Nobel Prize in 1928 for his work[8]. Finsen for his discovery that exposure to sunlight is effective in treating skin disorder got Nobel Prize in 1903[52].

Until late 1960's the knowledge about the chemistry of vitamin D did not advance, while the work of De Luca, Fraser, and Norman established vitamin D as a prohormone. It is converted to highly active forms by successive hydroxylation at 25 and 1 position. Vitamin D is both a hormone synthesized in skin and as a vitamin when taken from dietary sources[11].

BIOSYNTHESIS OF VITAMIN D:

In nature, Vitamin D are of two different forms:

- Vitamin D_2 and Vitamin D_3.
- Vitamin D_2 is derived from ergosterol called as ergocalciferol.
- Vitamin D_3 is called as cholecalciferol.
- When exposed to UV radiation (290 to 310nm) the cutaneous precursor of vitamin D, 7-dehydrocholesterol undergo photochemical cleavage of bond between carbon 9 and carbon10 of the steroid ring.

- The resultant previtamin D is thermally labile, over a period of 48 hours undergoes temperature dependent non-enzymatic molecular rearrangement resulting in production of vitamin D.
- This thermally labile product isomerizes to luminosterol and tachy sterol, two biologically inert products. This alternative photoisomerization prevents production of excessive amounts of vitamin D with prolonged sun exposure[23].
- Vitamin D_2 differs from D_3, double bond present between C_{22} and C_{23} and methyl group in C_{24} side chain[20]**.** And the two compounds have equivalent biological activity in humans and undergo identical metabolic conversion to active forms by hydroxylases[11].

SOURCES OF VITAMIN D:

Vitamin D_2 is provide by plant sources and vitamin D_3 is provided by animal sources . Sources are sun light, diet and supplements. Natural sources like cod liver oil, egg yolk, fish, mushroom and fortified foods like milk, butter, cheese, yogurts, orange juice are rich in vitamin D[41].

The mode of action of vitamin D can be elucidated by:

1. The endogenous activation of vitamin D is by sequential hydroxylation at C_{25} and C_1 .
2. Molecular mechanism subsequent to binding of 1,25dihydroxyvitamin D [$1,25(OH)_2D$] to a specific Vitamin D receptor (VDR) known to recruit large number of proteins.

3. Regulation of expression of genes involved either in homeostasis of calcium or linked to cell proliferation and differentiation

PHYSIOLOGY OF ABSORPTION, METABOLISM AND EXCRETION:

Absorption

Vitamin D taken in dietary form are fat soluble and it is absorbed principally in proximal small bowel[8]. About 80% of vitamin D is incorporated with chylomicrons and absorbed in lymphatic system. It enters the blood by a process involving binding to vitamin D binding protein majorly, although a small fraction is bound to albumin[18]. Defective absorption is seen in individuals with malabsorption syndromes like celiac disease, whipple's disease etc[11].

Metabolism of vitamin D:

Vitamin D from skin and diet can be stored in and released from fat cells[41]. It enters circulation by binding to a specific protein called vitamin D binding protein. Vitamin D is not active as such but it undergoes a series of metabolic transformations in the liver and kidney to form active metabolite[11].

Vitamin D metabolism involves three main steps:

Vitamin D is biologically inert and it undergoes consecutive hydroxylation in liver and kidneys to form 1,25$(OH)_2$ D, the active metabolite. The steps include

- 25-hydroxylation.
- 1α hydroxylation, and
- 24- Hydroxylation.
- By enzymes cytochrome p-450 mixed function oxidase located either in mitochondria or endoplasmic reticulum[19].

25 HYDROXYLATION:

The initial step occurs in the liver where vitamin D undergoes hydroxylation at 25th position by the enzyme 25- hydroxylase to form 25-(OH)D or calcidiol[5]. But it also occurs in extra hepatic tissues in vitro[8].

Hepatic 25-hydroxylation is performed by more than one enzyme located either in the microsomes (CYP2D11, CYP2D25, CYP3A4, CYP2R1) or in the inner mitochondrial membrane (CYP27A1)[12]. It is a mixed function oxidase for which NADP and molecular oxygen are utilized.

The most important 25- hydroxylase is CYP2R1. The 25 hydroxylation of vitamin D is not a regulated process. The principal circulating form is 25(OH) D. Its serum concentration is about 10 - 50 ng/ml and the half life is about 15 to 20 days[11]. It is the excellent measure of nutritional status of vitamin D because its levels increase in proportion with vitamin D intake[19].

CYP27A1 is the only mitochondrial 25-hydroxylase having broad substrate specificity and is mainly involved in hydroxylation steps in cholesterol and bile acid[12]. In liver disease circulating levels of 25-(OH) D is

reduced. Also in persons taking anti convulsant drugs like phenobarbitone and phenytoin reduced levels seen by inducing hepatic microsomal enzymes which inactivate vitamin D[11]. Homozygous mutation of CYP2R1 was identified in persons with classical rickets[13].

1 α HYDROXYLATION:

The second step in metabolic activation is 1α hydroxylation. 25(OH) D is inactive biologically and hence it should be hydroxylated further[10]. 1, 25$(OH)_2$ D or calcitriol is formed by hydroxylation in the kidney by 1α hydroxylase on the 1-carbon position of 25-(OH)D. The enzyme 1α hydroxylase (CYP27B1) is a mitochondrial cytochrome p450 mixed function oxidase. Requires NADP and ferrodoxin for formation of 1, 25(OH) $_2$D in proximal renal tubule.

The half life is about 4 to 6 hours. Because of its short half life and tight regulatory process 1, 25 $(OH)_2$ D is not a valuable marker to assess vitamin D status. 1α hydroxylase activity is noticed in other tissues like prostate, colon, breast, lung, pancreatic β cells, parathyroid, thyroid, testes, ovary, placenta, and cells of immune system lymphocytes and macrophages exhibiting autocrine and paracrine actions[20].

This process is a tightly regulated by 1,25$(OH)_2$D itself(negative indirect feedback) parathyroid hormone, calcitonin, and insulin like growth factor(positive feedback),phosphate, calcium, phosphorus and fibroblast growth factor 23 (FGF-23) negative regulation[14,15].

Parathyroid hormone (PTH) regulates 1α hydroxylase and mRNA activity in renal tubules mediated via cAMP by stimulation of 1α hydroxylase gene transcription. Restriction of dietary phosphate also known to elevate renal 1α hydroxylase activity and mRNA activity. Calcium from diet also regulates the activity of enzyme. Other factors like growth hormone, glucocorticoids, prolactin and estrogen also influence the renal 1α hydroxylase activity[11].

Mutations in 1α hydroxylase gene was reported in pseudo vitamin D deficiency rickets ,also called as vitamin d dependent rickets type -1 .It has low serum levels of $1,25(OH)_2D$,but normal to high levels of $25(OH)D$[8].

24-HYDROXYLATION:

25-(OH)D (Calcidiol) and 1,25(OH) 2D (Calcitriol) undergo hydroxylation at 24-carbon by the enzyme 24-hydrxylase(CYP24A1) to form 24, 25 $(OH)_2$ D and 1,24,25-$(OH)_3$ D in kidney, intestine and cartilage.

CYP24A1 has both 24-hydroxylase and 23-hydroxylase activity, the ratio is dependent upon the species. 1, 25$(OH)_2$ D is also metabolized to several inactive products by 23 or 26 hydroxylase.

The biologically inactive calcitroic acid is formed from the 24-hydroxylase pathway whereas the 23-hydroxylase pathway ends up in

producing the biologically active 1, 25-26, 23 lactone[25]. All steps are performed by one enzyme.

Calcitroic acid is the final degradation product that is excreted in urine**.** In addition polar metabolites are excreted in bile, some of these metabolites are deconjugated in intestine and reabsorbed in enterohepatic circulation[18]. No mutations have been identified in 24 hydroxylase gene so far but over expression of this gene may be involved in cancer[8].

TRANSPORT OF VITAMIN D:

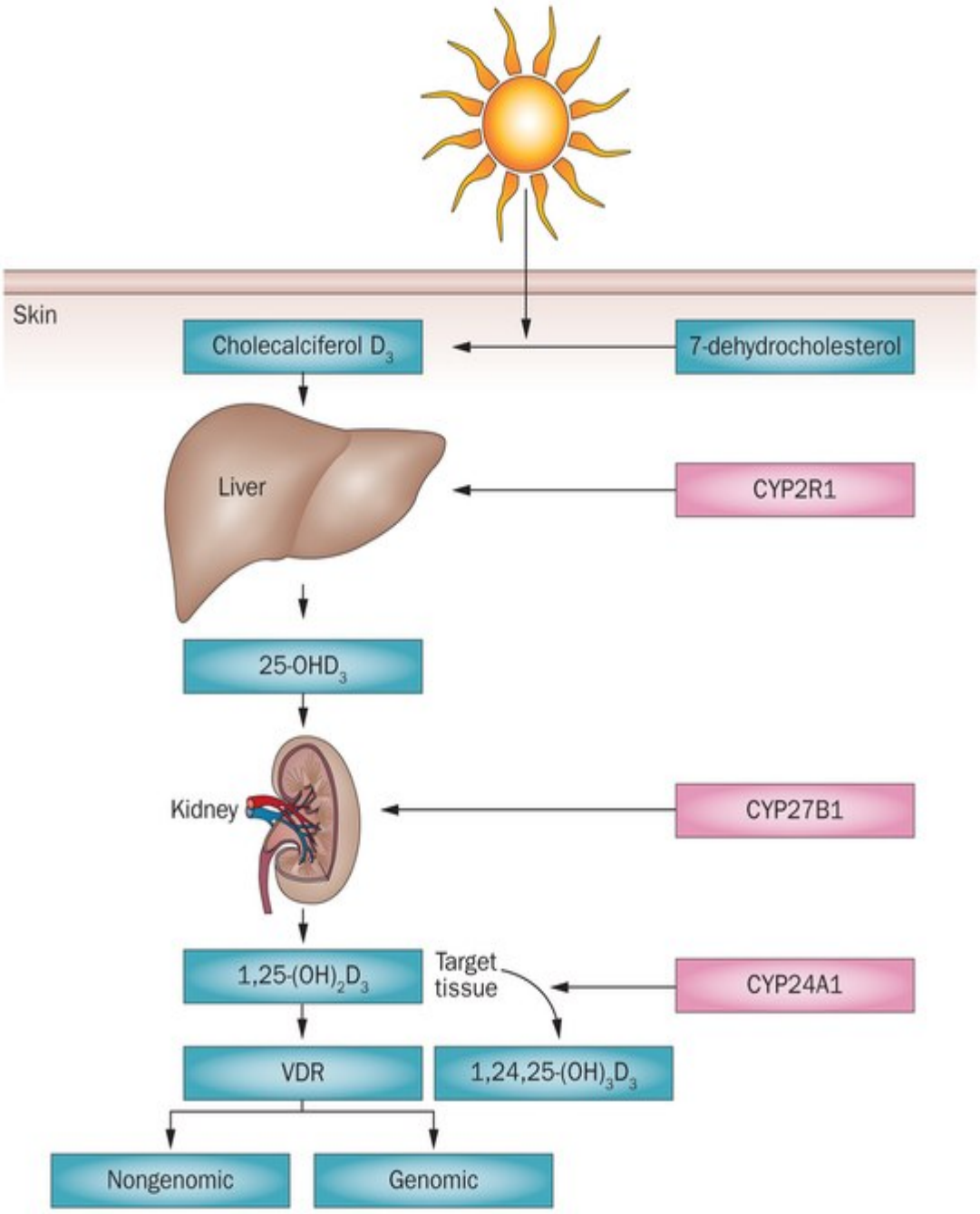
Vitamin D gets absorbed in gut and gets transported by chylomicron via the lymphatic system. It gets stored in tissues like muscle and fat[8].

Vitamin D is highly lipid soluble but it has poor solubility in aqueous media. By means of binding to a specific carrier protein called vitamin D binding protein (DBP) it gets transported in blood [26]. It gets hydroxylated and released as 25-(OH) D in liver.

EXCRETION:

The major route of excretion is through bile. Some of it is reabsorbed in the small intestine. Vitamin D is converted into more water soluble metabolites like calcitroic acid excreted by the kidneys via urine[8, 11].

FIG: 1 SYNTHESIS OF VITAMIN D



VITAMIN D BINDING PROTEIN:

DBP is the major carrier of vitamin D and its metabolites. It is a α globulin of about molecular weight 52,000 synthesized in the liver.

The protein has higher affinity for 25-(OH) D than 24, 25(OH) $_2$ D and 1, 25(OH)$_2$ D. Approximately 88% fraction of 25(OH) D circulates bound to DBP, 0.03% is free and rest bound to albumin. In contrast 85% of circulating 1, 25(OH) $_2$ D binds to DBP and 0.4%is free and rest binds to albumin[23].

The function of vitamin D binding protein is to retain the serum reservoir of vitamin D and modulation of activity of vitamin D metabolites. The function of DBP is to reflect the free hormone hypothesis which states that the free fraction unlike protein bound fraction of active vitamin D hormone is responsible for biological activity.

DBP concentration in plasma is 20 times more than the total vitamin D metabolites. Vitamin D binding with DBP have longer half life because they are less prone for metabolism in liver and biliary excretion. Unbound fraction of vitamin D is responsible for biological activity rather than protein bound fraction. The level of DBP is reduced in liver diseases, nephrotic syndrome and malnutrition while it is increased on estrogen therapy and its concentration doubles during pregnancy[8].

The cellular effects of 1, 25(OH) $_2$ D correlate with the free fraction. Vitamin D entry in to renal system by receptor mediated uptake of DBP in

brush border of proximal renal tubules but not by diffusion[24,26]. Megalin is a lipoprotein receptor present in the proximal tubule favors reabsorption of DBP and DBP complexed with vitamin D sterols[8].

FACTORS INFLUENCING CUTANEOUS VITAMIN D SYNTHESIS:

Vitamin D3 production is affected by the process which significantly alters the amount of UVB radiation. The wavelength optimum for the vitamin D production is 295 to 300 nm. This is influenced by factors like thick ozone , cloud and aerosol optical thickness, particulate pollutants, columns of water vapour, cloud cover, aerosols, latitude, altitude[28].

Solar zenith angle (SZA) also influence UVB radiation. It is the angle between the local vertical and observer line to the sun. Smaller SZA results in more intense UV radiation. Factors like time of day, latitude and season influence the zenith angle[28, 29].

UVB radiation, below latitude of 35° N is sufficient for vitamin D synthesis throughout the year. Vitamin D is not produced at higher latitudes during winter. Effective UV doses are equated to oral vitamin D_3 dose. 1 MED (minimal erythema dose) exposure of UV radiation produces a rise in serum vitamin D levels that is equal to that produced on oral vitamin D supplementation of 16,000IU.

Cutaneous factors like clothing, sunscreen and melanin also influence vitamin D synthesis. Cotton and linen blocks UV radiation compared to wool,

nylon silk and polyester[28]. Light pigmented persons require longer UV exposure times than dark skin people to produce equal amount of vitamin D.

Cellular basis of actions of vitamin D:

For most of the biological actions of 1, 25(OH) $_2$ D it requires a high affinity receptor, the VDR[24]. VDR is a transcriptional activator, a member of steroid hormone receptor super gene family. It modulates transcription of target genes. VDR is a phosphoprotein and phosphorylation is important for its function[11].

VDR binds 1, 25$(OH)_2$ D effectively at higher affinity even at sub nanomolar concentrations[35]. Regulation of transcription occurs either by direct binding of nuclear receptors with the specific response elements or influencing transcription by other DNA binding transcription factors. VDR expressed in almost all tissues from low to moderate levels but highest expression is seen in intestine, kidney, bone, skin and thyroid gland and regulates approximately 3% of human genes via its endocrine effect[34].

CLASSIC TARGET TISSUES:

The primary target tissues for the vitamin D endocrine system are by interactions among bone, intestine, kidney and parathyroid glands. Its role in mineral metabolism to maintain calcium levels is a complex interplay between calcium, phosphate, vitamin D, PTH , FGF-23.

PTH induces mobilization of bone calcium and stimulates production 1, 25(OH) $_2$ D by stimulating 1α hydroxylase activity. By negative feedback, $1,25(OH)_2D$ acts on parathyroid gland and inhibit secretion of PTH. By second negative feedback $1,25(OH)_2D$ limits its own availability by decreasing the activity of 1α hydroxylase enzyme while stimulating the enzyme 24- hydroxylase and induces its own catabolism.

FGF-23(Phosphatonin) is a phosphaturic hormone produced by osteocytes and osteoblast inhibits the activity of NPT2 gene, a newly identified target gene for $1,25(OH)_2D$. The NPT2 gene encodes sodium/phosphate co transporter essential for re absorption of phosphate[8].

CALCEMIC ACTIONS OF VITAMIN D:

IN INTESTINE:

The principle effect of vitamin D is induction of increased calcium absorption across the intestinal epithelial cells. Calcium is absorbed throughout the small intestine but the efficiency of absorption is highest in duodenum, as the receptors for 1, 25(OH) $_2$ D is found throughout the intestine[11]. Net absorption of calcium variesdepending upon dietary load of calcium, serum concentration of 1, 25(OH) $_2$ D and bioavailability of dietary calcium[8].

Acidification of food in stomach solubilizes calcium and in conditions like achlorhydria calcium absorption is decreased[31]. Finally the ability of

intestine to absorb calcium is decreases as age advances this may the reason behind the pathogenesis of osteoporosis[32].The process of vitamin D sensitive transport of calcium across the intestinal epithelial cell occurs by three steps[11]:

Vitamin D increases the uptake of calcium through the brush border membrane of intestinal microvilli. This is presumed to be the rate limiting step in the hormone sensitive transport of calcium.

1. The entry of calcium from the intestinal lumen into the enterocyte is regulated by the epithelial channels TRPV6 and TRPV5 (transient receptor potential vanniloid) the intracellular calcium transfer is by calbindin – D9K[8].
2. An ATP driven calcium pump in the basolateral membrane is capable of active extrusion of calcium into the blood against electro chemical gradient. Calcium exit could also be driven by a basolateral Na/Ca exchanger driven by sodium gradient.

EFFECTS ON BONE

Vitamin D is needed for growth of normal skeleton and bone , both in utero and childhood as well as for maintenance of skeleton in adults . 1,25 $(OH)_2$ D has dual effects on bone:

1. Stimulate osteoclastogenesis , bone resorption.
2. Modify osteoblast function and bone mineralization.

The overall effects of vitamin D on bone are more complex. From the observations made in humans it is clear that excess 1, 25(OH) $_2$ D enhance osteoclastogenesis and bone resorption while deficiency of vitamin D or resistance impairs bone matrix mineralization and osteoblastic activity is stimulated[8].

The coordinated actions of calcium and 1, 25 (OH) $_2$ D with VDR is required for growth plate development. $1,25(OH)_2D$ regulates osteoclastogenesis by reciprocal regulation of receptor activation of NF-kB ligand (RANKL) and osteoproteregin (OPG) Expression of RANKL on the surface of osteoblasts is increased by VDR[26].

RANK on immature osteoclasts binds to the receptor activator RANKL promotes the cells to mature and combine with other precursor osteoclasts to form mature multinuclear osteoclasts.

Bone remodeling, is an integrated process done by interactions between osteoclasts and osteoblasts. 1, 25(OH) $_2$ D, PTH and prostaglandins stimulates RANKL expression but and $1,25(OH)_2D$ inhibits osteoprotegrin production with increase in osteoclastic activity.

Deficiency of vitamin D causes secondary hyperparathyroidism resulting in decreased level of phosphorus. For mineralization of bone, calcium and phosphorus product should be adequate. Mineralization defect caused by low normal serum calcium and phosphorus concentration leading to rickets in children and osteomalacia in adults[13].

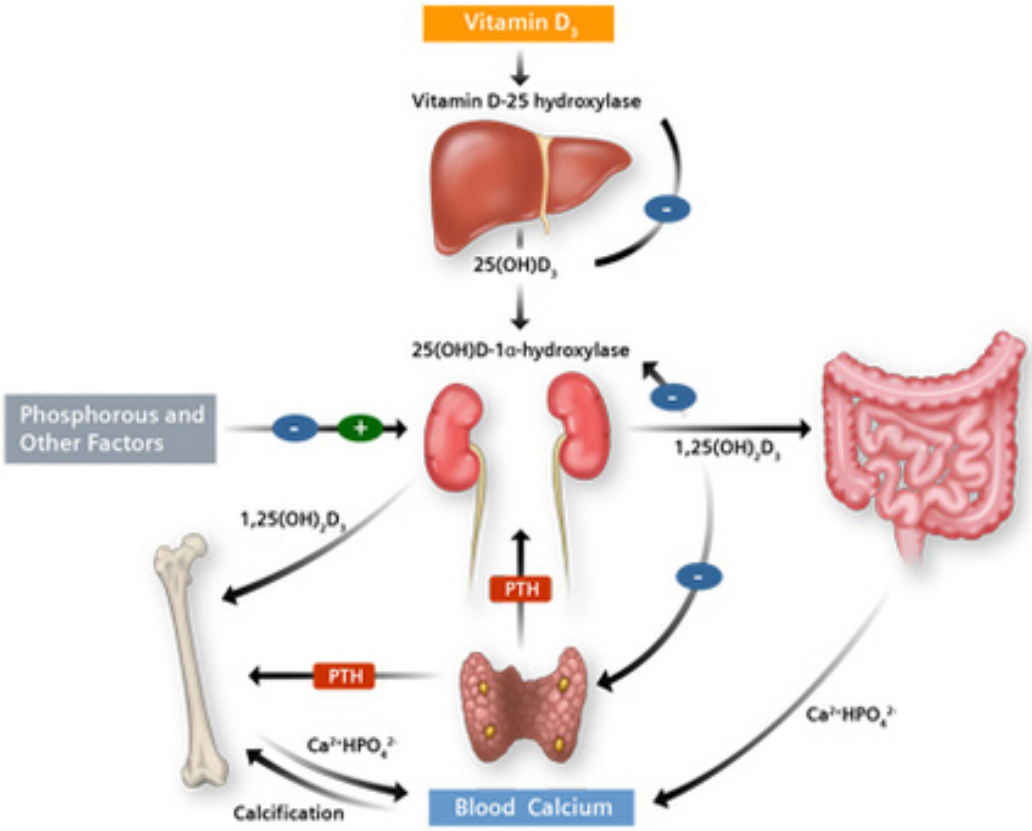
IN KIDNEY:

The kidney plays a major role in metabolism of 1, 25$(OH)_2$ D and reabsorption of calcium and phosphate .The proximal tubule of kidney is the central tissue for 1α hydroxylation of 25(OH)D. The 1αhydroxlase activity is reduced in chronic renal failure which ultimately results in renal osteodystrophy. 1, 25$(OH)_2$ D also increase the distal tubular reabsorption of calcium. TRPV5, Calbindin 9K and 28K and plasma membrane ATPase are involved[8].

1, 25 $(OH)_2$ D enhance phosphate reabsorption in proximal tubule of kidneys. It plays a role in phosphate homeostasis both PTH and FGF-23 in complex interplay with 1, 25 $(OH)_2$ D are able to reduce reabsorption of renal phosphate.

The effects of 1,25$(OH)_2$D on transport can be conceptualized as serving to maintain availability of phosphate for mineralization of bone by diminishing renal excretion and enhancing intestinal absorption. In Vitamin D deficiency calcium absorption is impaired. On giving vitamin D supplements it restores a normal threshold for renal calcium reabsorption and the renal tubule is sensitized to PTH [27].

FIG:2 CALCEMIC ACTIONS OF VITAMIN D



NON CALCEMIC ACTIONS:

Vitamin D is essential not only for maintaining bone and skeletal health but also play an important role in non skeletal biologic process. The virtual ubiquitous expression of VDR in all nucleated cells, the presence of 1α hydroxylase in different tissues like heart, brain, stomach, gonads, skin, pancreas, colon, placenta, vascular endothelium, and immune system apart from kidney.

And very large number of genes that are under direct or indirect control of $1,25(OH)_2D$ all point toward a more universal role for the vitamin D endocrine system than just regulation of calcium, phosphate, bone metabolism[8].

Vitamin D and cancer:

$1,25(OH)_2D$ has a potent role in preventing progression of cancer by reducing cell proliferation, promoting cell differentiation, apoptosis of cancer cells, reducing angiogenesis ,inflammation, and metastasis[18]. vitamin D plays a protective role in certain cancers like breast cancer, colon cancer, breast cancer.

The protective role of vitamin D in cancer is as follows[57]:

- It stimulates transcription of p21 which is a cyclin dependent kinase inhibitor acts by suppressing the cells of monocyte-macrophage lineage and promotes their differentiation.
- Vitamin D stimulates cyclin dependent kinase inhibitor p27.
- Vitamin D inhibit TGF (Tumor growth factor) which stimulates proliferation of tumor cells.

- Vitamin D and VDR prevents cancerous cell cycle progression at G1-G0 transition , which in turn reduces the rate of cell proliferation[24].
- It exerts anticarcinogenic effect by down regulation of Bcl-2 an anti-apoptotic gene and up regulates Bax, a pro-apoptotic protein and telomerase reverse transcriptase[41,40].
- Vitamin D inhibits proliferation of leukemic cell lines and thereby increasing maturation and decreasing aggressiveness of potential leukemic cells. Therefore, vitamin D is helpful in the treatment of leukemia.
- Other effects involve cancer cell death, block tumor growth, induces antioxidant mechanisms and repair of DNA[41].

  By regulating estrogen and androgen receptor signaling it inhibit the tumor growth of breast and prostate cancer.

Vitamin D and Cardiovascular disease:

The underlying mechanism by which vitamin D offer protection in development of atherosclerosis is by VDR present in vascular endothelial smooth muscle inhibit macrophage cholesterol uptake, decreased proliferation of vascular smooth muscle endothelial expression of adhesion molecules is reduced, and release of cytokines from lymphocytes also inhibited[41].

Endothelial dysfunction may be due to inadequate vitamin D intake, impaired vascular compliance, enhanced inflammation, or high levels of PTH and renin-angiotensin (RAS). The negative feedback of vitamin D on RAS plays a key role in regulating blood pressure, electrolytes and volume homeostasis[24].

Many prospective studies states, there exist an inverse relation with vitamin D levels and cardiovascular disease. Vitamin D could impact cardiovascular events either by VDR in smooth muscle vasculature or cardiac muscle or indirectly by promoting calcium absorption at the expense of lipid absorption or excretion in gut.

As shown in various epidemiological studies there is also an association between vitamin D and hypertension. On vitamin D supplementation or exposure to UV radiation there will be an increase 25(OH) D levels by increasing RAS activity which in turn have antihypertensive effects[24].

VITAMIN D AND DIABETES:

$1,25(OH)_2D$ having immunomodulator activity , implicated in prevention of type1and type 2 diabetes mellitus by reducing cytokine production, proliferation of lymphocytes, and destruction of beta cells of pancreas[38].

Autoimmune destruction of β cells of pancreatic islet cells results in Type-1 DM. The mechanism of action:

- Inhibition of dentritic cell maturation.
- Suppress the antigenic property of macrophages.
- Modulation of CD4 lymphocytes.
- Inhibition of interferon γ and interleukin2 production.

β cells of islets of langerhans express VDR and vitamin D stimulates the production of insulin secretion . Deficiency in vitamin D results in insulin resistance[38].

Obese persons having low 25(OH) D are more prone to get diabetes and metabolic syndrome.VDR expressed in adipocytes and 1, 25$(OH)_2$ D promotes lipogenesis while lipolysis is decreased[25].

Improving the status of vitamin D leads to increased insulin sensitivity. Children on vitamin D supplements have 30% reduction in risk of type1 diabetes as shown in observational studies[5].

Vitamin D and immune function:

Regulation of immune system by vitamin D is essential for protecting against infectious diseases. Presence of VDR in macrophages, dentritic cells, B cells and T cells regulates both the adaptive as well as innate immunity. On exposure to foreign antigens Vitamin D is capable of stimulating an antimicrobial peptide called cathelicidin. Thus cathelicidin plays a key role in innate immune defense mechanism and regulate the transcription of VDR gene[57].

1, 25$(OH)_2$ D in adaptive immune response acts by modulating the functions of dentritic cells. It inhibits maturation of dentritic cell and generates synchronized action by expression of cytokines like (IL-1, IL-2,IL-12,IL-17,IFN-γ) on T cells and the genes like(MHC class II and co signaling proteins) needed for antigen presentation . The global effect of these immune modulating action is thus a down regulation of acquired immune system[8]. T-lymphocyte proliferation is also inhibited and the expression of TH1 is shifted to TH2[36].

Vitamin D and skin:

The combined presence of vitamin D production, 25 hydroxylase, 1α hydroxylase and VDR expression in the epidermis suggest the existence of unique vitamin D intracrine system in which UVB irradiated keratinocytes may supply their own needs for $1,25(OH)_2$ D. The antiproliferative and prodifferentiation activity of VDR ligand is exerted on epidermal keratinocytes. The more differentiated keratinocytes acts a barrier by preventing water loss and protects from environmental microbes.

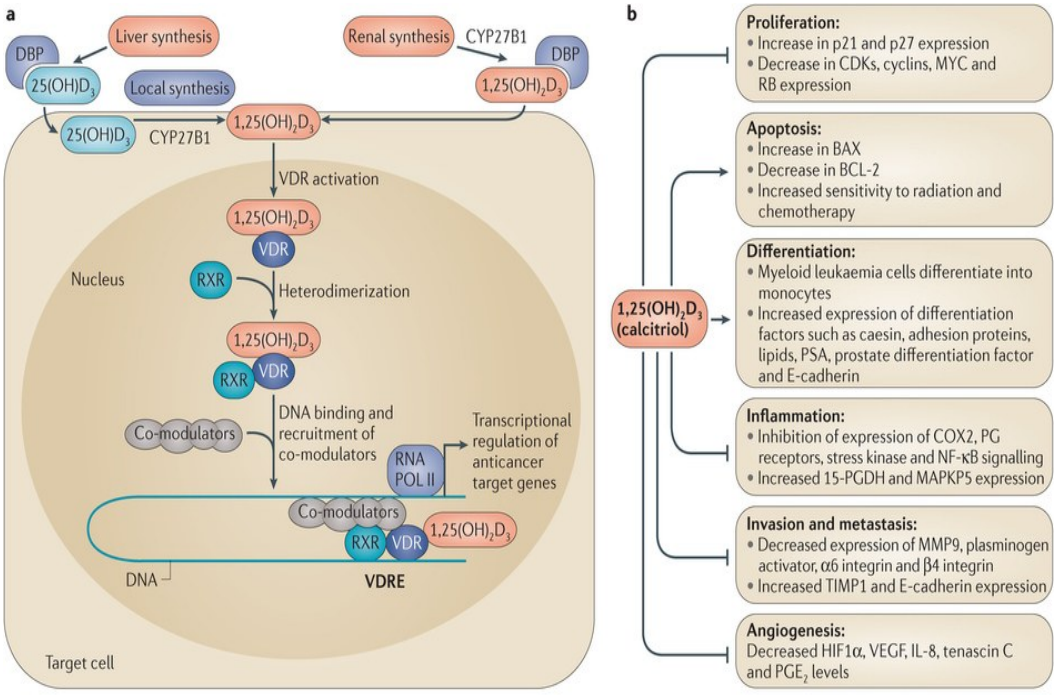
As cells differentiate they start to express keratins 1 and 10, as well as involucrin. In addition some proteins and lipids are expressed to form the cornified envelope which acts as a epidermal barrier. This barrier protects from toxins and infectious agents by acting as a host defense. The dermis is exposed to foreign antigens when the barrier gets disrupted. $1, 25(OH)_2$ D by its ability to inhibit proliferation and immune activity suppression have been used for treating psoriasis , a disorder of hyper proliferative and abnormal differentiation[25].

Chronic exposure to sun results in development of non-melanoma skin cancer by gene alterations, stress, and proinflammatory actions[16]. On supplementation with vitamin D there is a decrease in risk of recurrence of melanoma compared to those not treated with vitamin D as stated in Newton-Bishop et al.

Other diseases:

Risk of Depression , Alzheimer's disease, and Schizophrenia are high in vitamin D deficient persons. The risk of peripheral vascular disease is decreased by 80% in persons with sufficient vitamin D [24].

FIG:3 NON CALCEMIC ACTIONS OF VITAMIN D



ASSESSMENT OF VITAMIN D STATUS:

25(OH) D is the best diagnostic marker for assessing the vitamin D status and it may vary among different populations[8]. Because 25(OH) D is more stable and having half life of about 15-20 days being a most important key indicator of evaluating disorders of mineral metabolism.

Endocrine society and IOM (Institute Of Medicine) states vitamin D status based on levels of 25-(OH)D:

- vitamin D deficiency ------> < 10ng/ml
- vitamin D insufficiency ----> 21-29ng/ml
- Vitamin D sufficiency ------ > 30 ng/ml and the range being preferred is 40-60 ng/ml. Till 150ng/ml vitamin D toxicity does not occur[41]. Still, there exist a controversy in defining vitamin D deficiency or sufficiency based on 25(OH) D levels[19].

Other factor defining vitamin D status is suppression of parathyroid hormone. When 25 (OH) D levels are about 30ng/ml there exists stability in PTH suppression thus the rationale of selecting optimal vitamin D as 30ng/ml. There is evidence of low 25(OH) D, inspite of PTH level being normal and on the contrary PTH is not suppressed when 25(OH) D is increased[39].

Vitamin D functional status is also evaluated by 25(OH) D level that result in maximum calcium absorption from intestine.

Before labeling the person as vitamin D deficient PTH and calcium should be evaluated. Vitamin D level alone is not enough to predict the deficiency as PTH level is normal in 50% of population having decreased vitamin D levels[45]. A simplistic classification of vitamin D status is suggested in the Mayo Clinic proceedings 2011.

Classification of vitamin D status based on 25-(OH) D[8, 52].

≤10ng/ml ----→ Deficient

11-20 ng/ ml ----→ Insufficient

>20ng/ml -----→ optimal

EPIDEMIOLOGY:

The National Health and Nutrition Examination Surveys 2001–2006 had shown that 33% prevalence of vitamin D deficiency.

Vitamin D levels (<9 ng /mL) is prevalent in more than 35% of Indian school children. Over 80% of pregnant women in India had 25(OH) D levels <22.5 ng/ml[21].

Prevalence of hypovitaminosis D as per International Osteoporosis Foundation in North India are neonates 96%, 91% of healthy school girls, 78%of hospital staffs and 84% of pregnant women. In South India the prevalence of vitamin D deficiency is 40% in males and 70% in females[45].

Modernization, evolutionary changes, clothing, hours spent indoors, obesity, environmental pollution, and use of sunscreen result in reduced vitamin D synthesis[33].

ASSAY FOR VITAMIN D AND METABOLITES

There are many methods available commercially for determination of vitamin D status. 25(OH) D and 1, 25(OH) $_2$ D are the two metabolites used for assay of vitamin D status. Although 1, 25(OH) $_2$ D is the most active form it is not measured but assay of serum 25(OH) D is the choice because it is the major circulating metabolite, having longer half life and it reflect the vitamin D status[38]. This measurement helps for monitoring vitamin D therapy.

<u>The assay for 25(OH) D[53]:</u>

- High pressure liquid chromatography (HPLC).
- Liquid chromatography Mass Spectroscopy
- Radioimmunoassay(RIA)
- Enzyme immunoassay (EIA)
- Competitive protein binding assay(CPBA)
- Automated chemiluminescent protein binding assay(CLPBA)
- Chemiluminescent immunoassay (CLIA).

The most commonly used assays to determine vitamin D status are radioimmunoassay and chemiluminescence protein binding assay. Among various assays available, variability exist between different assays among laboratories even when using the same assay resulting in false high or

low values. To improve the variability among methods, a standard reference material is available for standardization of values across labs to enhance accuracy and reproducibility[38]. The widely accepted reference method for 25-(OH)D assay is LCTMS (liquid chromatography tandem mass spectrometry).

RECOMMENDED DAILY INTAKE OF VITAMIN D (RDA):

All the breast fed infants should receive 400 IU/day of vitamin D supplements as recommended by American academy of pediatrics. Non breast fed infants have to receive 400IU / dayof supplements until they receive Vitamin D fortified milk. The RDA for children and adolescents are approximately 600IU/day recommended by Food and Nutrition Board[54].

The serum level of 25(OH)D increases by 0.6-1.0 ng/ml for every 100IU/day. The IOM(Institute of Medicine) recommends the increase of 25-(OH)D to 20ng/ml for maintaining adequate bone health[41].

Recommended Dietary Allowances (RDAs) for Vitamin D

Age	Male	Female	Pregnancy	Lactation
0–12 months*	400 IU	400 IU		
	(10 mcg)	(10 mcg)		
1–13 years	600 IU	600 IU		
	(15 mcg)	(15 mcg)		
14–18 years	600 IU	600 IU	600 IU	600 IU
	(15 mcg)	(15 mcg)	(15 mcg)	(15 mcg)
19–50 years	600 IU	600 IU	600 IU	600 IU
	(15 mcg)	(15 mcg)	(15 mcg)	(15 mcg)
51–70 years	600 IU	600 IU		
	(15 mcg)	(15 mcg)		
>70 years	800 IU	800 IU		
	(20 mcg)	(20 mcg)		
* Adequate Intake				

Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: National Academy Press, 2010

CLINICAL CONSEQUENCES OF VITAMIN D DEFICIENCY:

SKELETAL:

1. Rickets is known to occur in children of age less than 2-3 years. It is characterized by defective mineralization of the skeleton in the growth plate and metaphysis adjacent to it.

Symptoms like pain in bones of legs, delayed age of standing or walking, delayed growth, and frequent falling. On physical examination – enlarged anterior fontanalle, frontal bossing, craniotabes, delayed tooth eruption, widening of metaphysis (enlargement of wrist and ankles) are seen[8].

Diagnosis should be confirmed by X-ray, showing osteopenia, cupping and fraying of metaphysis, and fuzziness of zone of calcification. These changes are seen in active growth plate namely distal ulna and femur, proximal and distal tibia. Biochemically serum alkaline phosphatase level will be elevated, phosphorus will be low or normal, calcium will be low and 25(OH) D will be <5ng/ml[39].

2. OSTEOMALACIA:

Defective mineralization of the mature skeleton. The un mineralized osteoid will be deposited at sites other than metaphysis is referred as osteomalacia. Bone pain is the characteristic symptom. Proximal muscle weakness and instability of gait are the other symptoms. X ray show pseudo fractures of pelvis, metatarsals, femur and scapula. Biochemical feature is elevated alkaline phosphatase, PTH, decreased calcium, phosphorus and vitamin D[39].

3. Osteoporosis,

4. Increased risk of fracture hip and vertebra.

NON SKELETAL[38]:

- Respiratory diseases like influenza, tuberculosis and chronic respiratory disease such as cystic fibrosis, interstitial lung disease and chronic obstructive lung disease.
- Cardiac hypertrophy, myocardial infarction, stroke, cardiac failure
- Autoimmune encephalomyelitis, thyroiditis , rheumatoid arthritis.
- Renal fibrosis
- Diabetes mellitus type 1& 2
- Cancer of breast, colon and prostate, leukemia and other myeloproliferative disorders.
- Multiple sclerosis.
- Mental illness.

VDR STRUCTURE AND FUNCTION:

VDR belongs to the family of nuclear hormone receptor super family. It plays a role in maintaining calcium and phosphorus homeostasis, regulation of cellular proliferation, mediates apoptosis and modulate immune responses. It acts a ligand dependent transcription factor that is able to activate or repress target genes by inducing conformational change within the receptor. VDR enhances heterodimerization with RXR (retinoid X receptor) to form VDR –

RXR heterodimer and it induces binding with vitamin D responsive elements (VDREs) which is located at the promoter region of target gene .

VDR having molecular mass of approximately 48 kDa consist of 427 amino acids. Based on function it is divided into various domains[59].

- **A/B domain**: Highly variable domain present in the amino terminus having 20 amino acids. Concerned with ligand induced VDR transactivation.
- **C domain**: is the DNA binding domain present between 21 to 92 amino acids.
- **D domain**: is the flexible hinge region located from 93 to 123 amino acids connecting the DNA binding domain and the ligand binding domain..
- **E domain**: is the ligand binding domain between amino acids 124 to 427. Dimerization and transactivation are concerned with this domain.
- **F domain**: not conserved domain.

STRUCTURE OF VDR:

A/B DOMAIN:

This domain is short compared to other member of nuclear receptor family. It is concerned with transcriptional activation function. Certain co activators (p160) and DRIP complexes interact with A/B domain and induce domain mediated transactivation. The receptor present in the A/B domain contributes for constitutive ligand independent transactivation, activattion Function-1(AF-1). The ligand binding, DNA binding or transactivation of

A/B domaim will not affect the autonomous transcription activation of A/B domain[58].

DNA binding domain[59]:

It is the most conserved domain. It consists of highly conserved amino acid residues ($C_{24}$to M_{89}) and 44 variable residues (F_{16} to I_{23} and M_{90} to S_{125}).

It have two zinc finger motifs each of it is having four cysteine residues coordinated tetrahedrally with zinc atoms. Zinc and cysteine residues are necessary for maintaining three dimensional structure of DBD. Zinc finger motifs form the core structure of DBD and each core is composed of two helices (helix1 and helix 2).

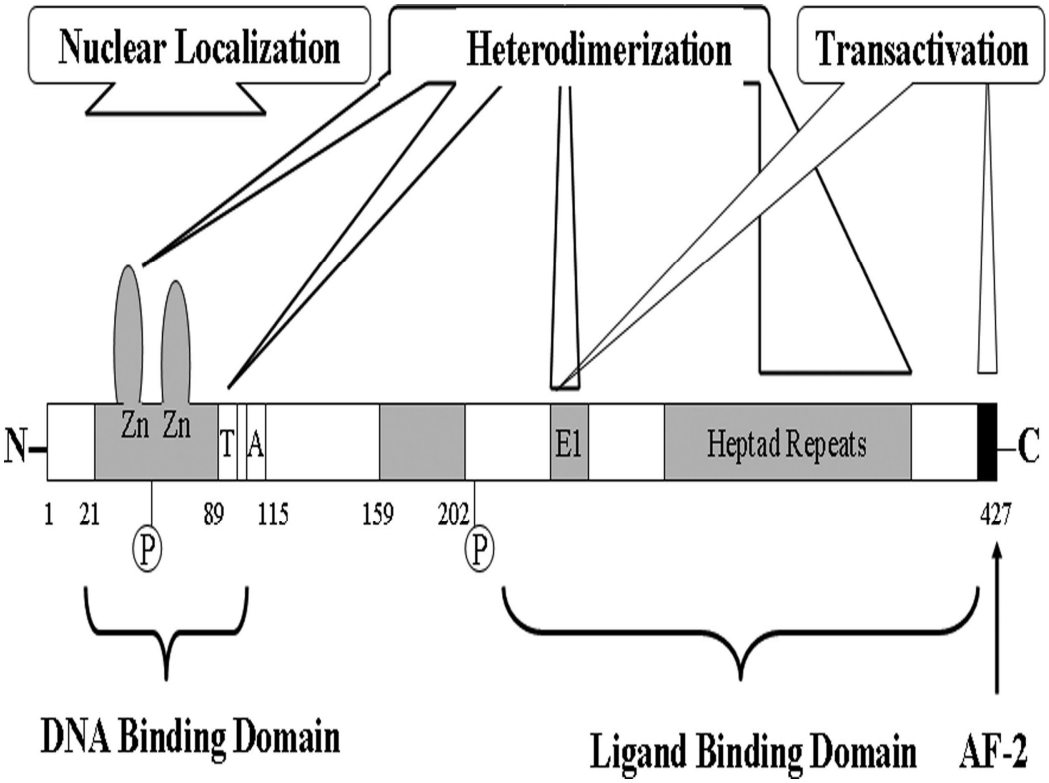
These two helices are oriented at right angles to each other and each serves separate functions.

Helix1 is concerned with specific binding to the major groove of hormone response elements on DNA and helix-2 for receptor dimerization .

FIRST ZINC FINGER MOTIFS[59]:

- Made of 22 amino acid residues (C24 to K45). Constitutes P box and S box. It is involved in carrying out two functions namely dimerization and VDRE binding.
- Recognition helix is formed by P box.
- R49 and K53 referred as S box.
- S box is involved in both heterodimerization and VDRE binding.
- Formation of dimer with RXR by both N37 and S box.
- V26, R50, P-box, S-box participate in binding with vitamin D response elements.

FIG:5 FUNCTIONAL DOMAINS OF VDR



SECOND ZINC FINGER MOTIFS:

- Consist of 21 amino acid residues (C60 to R80) and adjacent amino acids (Q77 to R80), (L81toI87). Of which the aminoacid residue forming structure of VDR homodimers are P61, F62, H75.

- The three residues forming upstream VDR are P61(Pro61), F62 (Phe62) and H75(His75).

- The downstream of VDR N37 (Asn37), E 92(Glu92), F93 (Phe93) form DBD interface. They are forced together by vanderwaals forces.

- The VDR DBD was unable to interact with VDIR (VDR interacting repressor)in mutation where proline replaced by threonine. D box of second zinc finger motifs causes transrepression of VDIR having weak dimerization function.

C-Terminal Extension of VDR DBD:

Consists of 36 amino acid residues (M90 to L95) and it is the addition of VDR DBD. The contiguous 6 amino acid residues (M90 toL95) is called as the T box. It is limited to form dimerization interface for VDR- RXR interaction to occur. Of the six amino acid residues of T box K91 and E92 form salt bridges with D39 and R38 in RXR, while F93 and I94 takes part in all functions of CTE.

LIGAND BINDING DOMAIN (LBD)[55] :

LBD is a multifunctional domain involves:

- Binding of 1,25$(OH)_2$D ligand
- Interaction with co repressors and co activators
- Strong heterodimerization with RXR.

The ligand binding induces conformational change that enhances dimerization and transactivation interfaces. All these together are essential for regulation of transcriptional activities.

Analysis by three dimensional structures indicates that LBD forms three structures based on the ligand binding state of receptors.

1. Apo receptor without ligand.
2. Holo agonist and antagonist receptor with agonist ligands
3. Holo agonist and antagonist receptor with antagonist ligands.

The receptor binding induces structural changes within the ligand binding domain. The most striking conformational change is seen in C-terminal helix-12, is the ligand dependent activation function (AF-2), it gets repositioned on ligand binding. In Apo receptor the ligand binding cavity is open and the helix-12 is exposed whereas in holo receptor the helix-12 will be rotated back. In addition minor structural changes will be seen in helices H3,

H6, H11. These changes produce receptor by disrupting binding of co repressors and generate surfaces for interaction with co activators and activate transcription.

Antagonistic ligands bind in the same ligand binding pocket as agonist ligands. It prevents correct assembly of helix 12 and disrupts binding of coactivators and inactivates transcription[58].

VDR is unique among other nuclear hormone receptor super family (NHR). Unlike NHR it contains large insertion domain connecting H1 and H3 and encoded by additional exon in VDR gene.

The crystal structure of LBD describes that helix-12 is critical for ligand binding and transactivation. H12 is stabilized by hydrophobic amino acid residues of helices H3, H5, H11. This in turn stabilized by two polar interactions. One is by salt bridge betweenLys-264(H4) and Gl5u-420 and other is hydrogen bond between Ser-235(H3) and Thr415. Some of the residues which stabilize H12 also interact directly with 1,25$(OH)_2$D (Val-234, Ile-268, His-397, and Tyr-401) .This explains that position of H12 is controlled by1,25$(OH)_2$D.

VITAMIN D RESPONSE ELEMENTS:

High affinity interaction between DBD of VDR with specific DNA sequences in the promoter region of 1, 25$(OH)_2$D target genes called as vitamin D response elements (VDREs)[24]. It can be classified as positive

VDREs (DR3 and DR4type and ER9) and negative VDREs (DR3 n VDRE and 1α n VDRE).

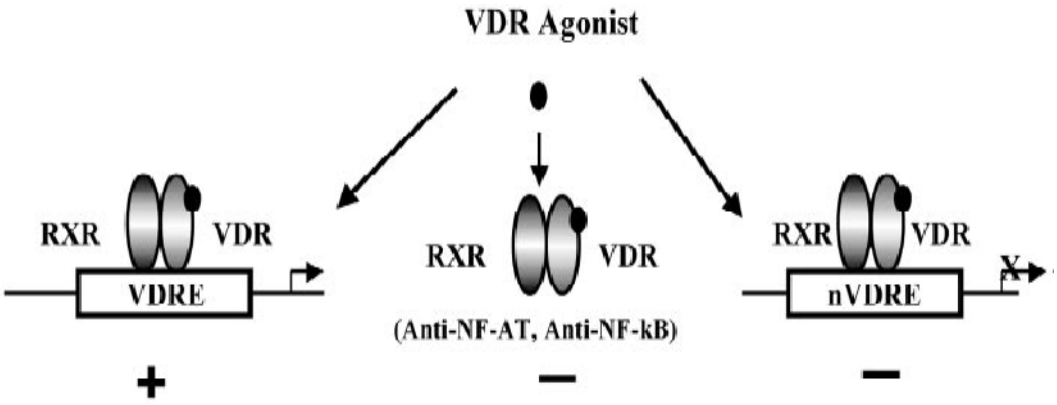
VDREs of positively regulated genes are direct repeat of two hexameric binding motifs separated by a spacer of 3 or 4 nucleotides (DR3 and DR4). Or they may be the everted repeats separated by a spacer of 6 or 9 nucleotides namely ER6 and ER9 respectively.

DR3 is the cognate VDRE and is the common type. It contains consensus sequence 5′-AGGTCA-3′, separated by a spacer of 3 nucleotides to which RXR-VDR bind the 5' half site and 3' half site respectively form heterodimer and target VDRE structure[8].

DBD does not form dimer in the absence of target DNA .On binding to ligand dimer gets strengthened. Unliganded VDR forms homodimer on binding to response elements. VDR forms heterodimer with RXR on binding of ligand.

Parathyroid hormone-related peptide (PTHRP) gene is also mediated by VDR-RXR heterodimer, down-regulatory by binding directly to a 16-bp sequences which is almost perfect repeat separated by a 3-bp nucleotides [59]. A non-consensus(GGGTGGA) direct repeat is located in 3-bp upstream of this motif, and it is called DR3-liked nVDRE

Fig.5 VITAMIN D RESPONSE ELEMENTS



Gene	Protein function	Anti-Inflammation	Anti-Proliferation	nVDRE
Osteocalcin (20)	Bone matrix protein			
Osteopontin (22)	Bone matrix protein			
RANKL (24, 25)	Bone remodeling	IL-2 (34, 35)	EGF-R (38)	PTH (43)
CA II (23)	Bone remodeling	IL-12 (36)	c-myc (38)	PTHrP (44)
Calbindin-9k (27)	calcium binding	TNF-α (35)	K16 (39)	Rel B (15)
24-hydroxylase (30)	Metabolism	IFN-γ (35)		
mCYP3A11 (32)	Metabolism	GM-CSF (37)		
rCYP3A1 (32)	Metabolism			
hCYP3A4 (12)	Metabolism			
β3 integrin (26)	Adhesion			
P21 (29)	Anti-proliferation			
Involucrin (31)	Differentiation			
PLC γ1 (31)	Differentiation			
IGFBP-3 (33)	Anti-proliferation			

Another novel nVDRE is 1α nVDRE is involved in down regulation of 25(OH)D 1α hydroxylase(CYP27B1)gene by 1,25$(OH)_2$D. Here the VDR-RXR donot bind directly. By means of VDIR (VDR interacting repressor) another transcription factor, helps in binding of liganded VDR- RXR with 1α nVDRE[34].

Recently, based on VDR interaction with co regulators provides new insights for positive and negative modulation of VDR mediated transcription.

Two domains serve as adaptor surfaces necessary for VDR-co regulator interactions.

1. Region located between residues 244 and 263. This residue form part of binding interface with transcriptional co activators. It is highly conserved residue. Its alteration compromises transactivation.
2. AF-2 or Activation Function corresponds to the helix 12 (residues 416-422). It undergoes conformational change on ligand binding and allows recruitment of VDR interacting proteins like RNApolymeraseII, Transcriptional initiator complex and nuclear transcriptional coactivators. Removal of AF-2 domain eliminate the transcriptional activity of 1,25$(OH)_2$D-VDR transcriptional activity[24].

TRANSACTIVATION[55]:

The regulation of gene transcription involves three classes of proteins:

1. Basal transcription factors → serve to initiate transcription of protein coding genes.
2. DNA binding transcription factors → precise control of transcription by VDR.

3. Transcriptional coactivators → provide protein –protein contacts between basal transcription factors and DNA binding factors

Transcriptional coactivator complex involved in nuclear receptor mediated transcription mechanism are:

1 → ATP dependent chromatin modeling

2 → Interaction with transcription factors and RNA Polymerase II

3 → modification of histone tails [36].

MOLECULAR MECHANISMS OF VDR IN THE CONTROL OF GENE TRANSCRIPTION:

1, 25(OH) $_2$ D interacts with VDR, initiates the series of events which either activates or repress gene transcription. The nucleosomal repression is released and gene transcription is initated.

COVALENT MODIFICATION OF DNA:

Transcription of genes is repressed by dense packing of DNA by limiting the access of transcription factors and RNA polymerase II to the binding site. Covalent modification of lysine at the amino terminal histone tail neutralize their positive charge thereby the negatively charged DNA attraction towards it is neutralized.

The binding of VDR to the hormone response elements in the promoter region of vitamin D response genes is the initial step for transactivation. Nuclear hormone receptors have the ability to bind DNA although it is wound around histone core. Ligand binding to hormone receptor induces conformational change in the DNA there by releasing nucleosomal

repression. It promotes receptor dimerization and creates new surfaces on the receptor for binding coactivator molecules to initiate the cascade of transcription events.

CO ACTIVATORS:

Two steps to be regulated by co activators in target gene transcription:

1. Remodeling of chromatin structure to facilitate binding of other co activators and RNA Polymerase II.

2. Recruiting protein complexes that interact with subunits of RNA Polymerase II by stabilizing the preinitiation complex[58].

Two enzyme complexes plays role in transactivation are:

- Histone Acetyl Transferases (HAT)

- ATP dependent chromatin remodeling factors.

HISTONE ACETYL TRANSFERASES (HAT)

Histone acetyl transferases modify the structure of chromatin by catalyzing acetylation of the lysine residues present in the N-terminal tails of histones. These include steroid receptor coactivators (SRC) family members like SRC-1, SRC-2, SRC-3, CBP(CREB binding protein/p300) and pCAF (p300/ CBP associated factor) Acetylation of histones is a dynamic process. The mechanism possible for the reversible activation and repression of transcription is because of hyper and hypo acetylation of lysine residues. Hyper acetylating histones within transcriptionally active chromatin have

rapid turnover half life of few minutes whereas the hypo acetylating histones of transcriptionally silent regions have less rapid turnover.

SKIP (SKI-interacting protein)/ nuclear receptor(NR) coactivator (NCoA)-62 synergized with SRC-1 and SRC-2 to induce RXR-VDR mediated ligand dependent transactivation[58].

SRC family members, CBP/p300, NcoA-62, TATA binding associated factors act as transcriptional coactivators and strongly potentiate ligand dependent activation of transcription by VDR.

ATP MEDIATED CHROMATIN REMODELLING FACTORS:

The co activators like SWI/SNF, ISWI, and Mi-2 are involved in remodeling of chromatin catalyzed by uncoupling of ionic interactions between histones and DNA by ATP hydrolysis.

This complex is involved in initiation of transcriptional activation by altering the chromatin which facilitate tight binding of NHR to the hormone response elements makes the histone tails accessible to the HAT complexes. Also mediate final event in initiation process by binding of transcriptional factors to the DNA template. In addition this complex mediates transcriptional repression by changing the conformation of chromatin from open state to closed state[58].

MEDIATOR COMPLEXES:

TRAP/ DRIP (vitamin D receptor interacting protein) are the mediator complexes which recruit components to the preinitation complex by acting as

a link between ligand activated receptor and RNA polymerase II instead of modifying the structure of chromatin.

COREPRESSORS

NcoR-1, NcoR-2, and hairless are the co repressors that engage in histone deacetylase activity. Then it deacetylate the lysine residues present in the N-terminal portion of histone tails. It suppresses transcription by chromatin compaction and gene silencing[42].

REGULATION OF VDR MEDIATED GENE TRANSCRIPTION:

VDR is the ligand dependent transcription factor that can modulate transcription of VDRE responsive genes in three different ways:

1. It can positively regulate the expression of certain genes by binding to VDRE present in their promoter regions.
2. It can negatively regulate the expression of genes by binding to negative VDRE.
3. Inhibit the expression of genes by antagonizing the action of transcription factors such as NF-AT, NF-κB.

Genes that contain VDRE in their promoter regions are: osteocalcin, osteopontin, RANKL and carbonic anhydraseII involved in extracellular bone matrix formation and bone remodeling.

Genes that contain VDRE in their promoter region and show vitamin D dependent up regulation in their expression are β3integrin, p53, calbindin9k, 24-hydroxylase etc. Genes that are negatively regulated by vitamin D are by two mechanism.

1. By binding to n VDRE in promoter region. Example PTH and PTH-RP
2. Bytranscription factor antagonism (AntiNFκB and AntiNF-AT).This is responsible for the anti-inflammatory and antiproliferative actions of vitamin D.

The genes having anti inflammatory actions are IL-2, IL-12, IFNγ, TNFα, GM-CSF. And those having anti proliferation actions are EGF-R, C-myc, K19.

The classic VDR bound with 1, 25(OH) $_2$ D can initiate both traditional genomic response as well as non-genomic response which is rapid signaling cascade.

FIG:1 B illustrates how hormonal ligand could be influencing VDR to interact efficiently with its partner RXR , VDRE and with coactivators. The steps involved are:

1. 1, 25$(OH)_2$ D_3 ligand present in the VDR genomic pocket (GP) produces a conformation which creates a cleft in the position of helix 12 at the C-terminus of VDR by bringing it to the "closed" position thus rendering the nuclear receptor to interact with coactivator through their NR boxes[42].

2. These co activators are attracted towards the helix 3,5,12 of liganded VDR which in turn allosterically stabilizes VDR-RXR heterodimer on VDRE and aid in triggering strong heterodimerization[46].
3. Hence VDR LBD is migrated towards the 5'side of RXR LBD and in doing so RXR LBD is rotated to 180˚ by the driving forces of ionic and hydrophobic interactions between 9 and 10 helices of VDR and consequent helices of RXR.
4. 1, 25$(OH)_2$ D mediated gene transcription the events like ligand intensified heterodimerization, VDRE docking, and coactivator recruitment by VDR appears to occur always together and are not separable
5. These conformational changes allosterically elicited in VDR by the interactions with ligand, RXR and DNA have the extra effect of converting VDR into more capable substrate for one or more serine protein kinases.
6. The most prominent phosphorylation is catalyzed by casein kinase II on VDR serine 208. This event potentiate the transcriptional activity of RXR-VDR heterodimer possible by enhancing interaction with coactivators DRIP 205.
7. The liganding of VDR conformationally influences its RXR heteropartner and cause the AF-2 region of RXR to rotate into the closed or active position.

8. Now RXR have the potential to bind additional coactivator and serve as a subordinate partner where VDR is the primary receptor within the heterodimer. Hence RXR on heterodimerization with VDR cannot bind 9-cis retinoic acid[46].

LIGAND DEPENDENT REPRESSION:

1. One of the mechanisms of repression is recruitment of co repressors in the vicinity of target gene. This is done by altering the chromatin architecture which is catalyzed by acetone deacetylases and methylases towards the receptor tethered corepressors.
2. The initial targeting of repressed gene is hypothesized by docking of liganded VDR-RXR on negative VDRE.
3. In case of repression the liganded VDR is conformed such that it binds to co repressor rather than co activator.
4. Non consensus nucleotides in negative VDRE occur in either or both half elements, such base pair changes may be adequate to drive RXRVDR into reverse polarity on negative VDRE. So that the corepressor is recruited to the helices 3 to 6
5. VDR undergo modification by protein phosphatases which yet again favor for co repressor attraction.
6. VDR-RXR heterodimers on interaction with VDIR at the E- box of negative VDRE comprising CANNTG –like motif. These interactions promote co regulator switching so that co activators p300 is disassociated and co repressors like histone deacetylator gets associated resulting in transrepression.

7. Other mechanism involves WINAC (Williams syndrome transcription factor including nucleosome assembly complex) induces transrepression by interacting with ATP dependent chromatin remodeling complex and VDR induced DNA methylation.

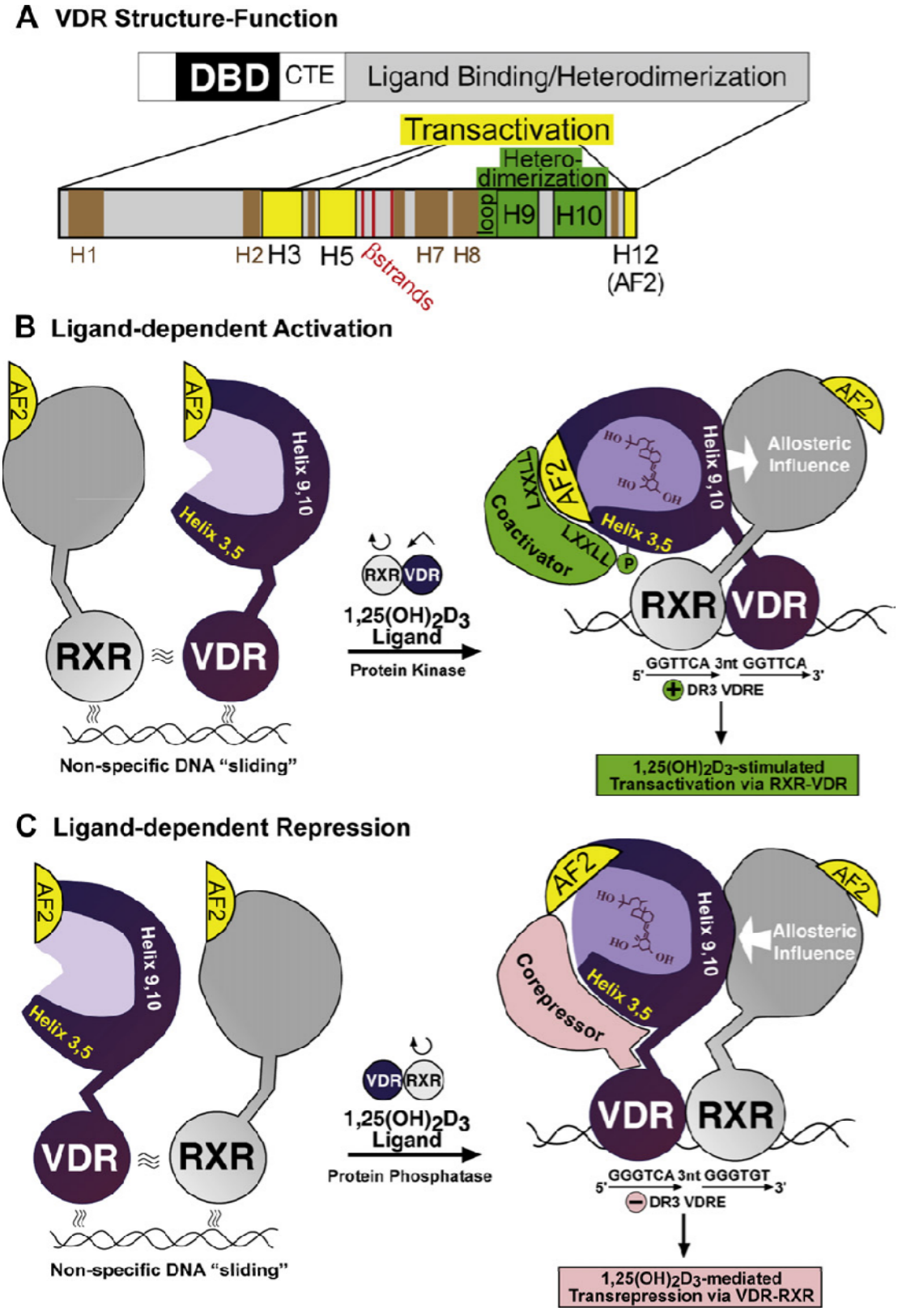
8. Epigenetic mechanism is also involved in negative regulation of VDR. Example 1α hydroxylase gene is repressed by FGF-23 and1, 25(OH) $_2$ D, while the latter regulated by epigenetic demethylation.

9. An emerging mechanism for control of gene expression is modulation by specific micro RNAs.[26]

VDR mediated non-genomic responses:

1, 25(OH) $_2$ D by its strong non covalent binding to its cognate receptor also elicit rapid response in target cells. Non-genomic signaling is rapid, and responses are generated within 1-2 min to 15 to 45 minutes rather than genomic takes hours to days to be apparent fully. It may indirectly affect transcription by cross-talk with other signaling pathway. It involves a non-classical membrane receptor for 1,25$(OH)_2$D called 1,25D3-MARRS (membrane-associated, rapid response steroid-binding).

Fig.6 VDR MEDIATED GENOMIC EFFECTS



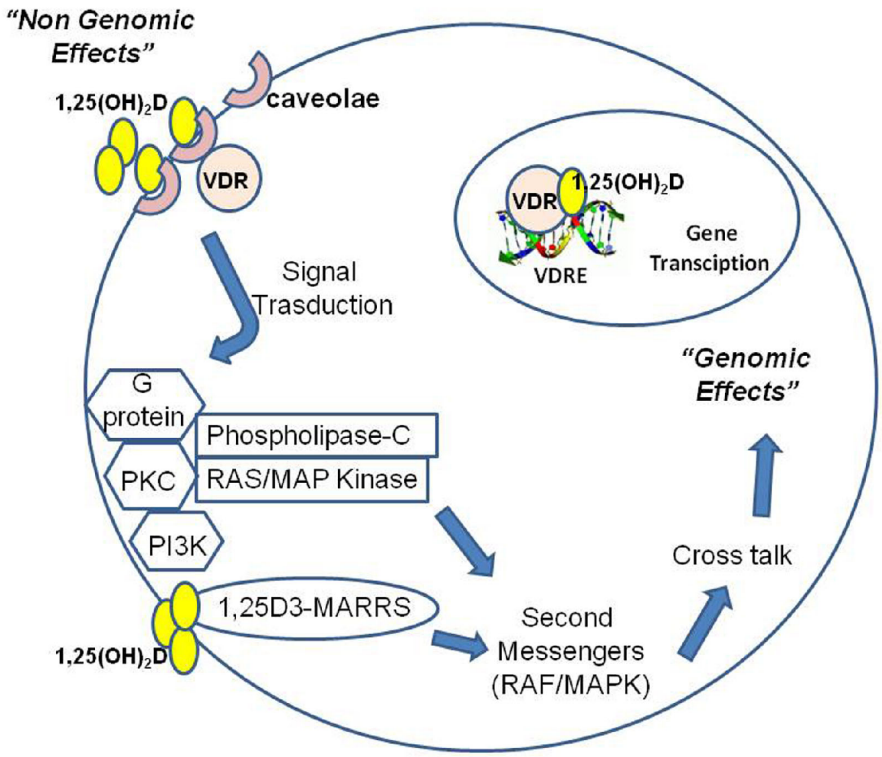
Non genomic actions induce[36] :

- Calcium is translocated across the mucosal membrane of intestine
- Second messengers like phospholipase C, protein kinase C(PKC), G-protein coupled receptor or phosphotidylinositol3kinase(PI3K) are activated when 1,25$(OH)_2$D is bound to the plasma membrane
- Activation of those second messengers causes voltage gated calcium or chloride channels to be opened.
- RAF/MAPK, is also a second messenger that may modulate by cross-talk with the nucleus for regulation of gene expression .
- Transcriptional activity of VDR is improved by ERK.
- PKC stabilize VDR via phosphorylation .

In various cell types, rapid responses can be mediated by 1,25-(OH)2 D acting through a population of classical VDR molecules acting outside the cell nucleus, associated with caveolae of plasma membrane[46].

VDR with ligand of its shape planar, localized in the caveolae of plasma membrane activates signal transduction pathway which generate rapid responses like generation of calcium or secretion of insulin via PKC or MAP Kinase to get activated by which chloride or calcium channels get opened or exocytosis.

Fig.7 : NON GENOMIC EFFECTS



When the genomic pocket of 1, 25$(OH)_2$D is occupied by bowl shaped ligand VDR interacts with RR to for heterodimer that binds to VDREs in the region of genes controlled directly by 1,25$(OH)_2$D. By recruiting coactivators or co repressors VDR-RXR-DNA modulate transcription of genes encoding proteins that exhibit genomic functions of vitamin D.

Overview of polymorphism:

It is of great interest in genetic studies which provides the opportunities to link molecular insights with the epidemiological data. Variations in the sequence of DNA or gene which occur in at least 1% of population is termed as "polymorphisms". It produces modest and subtle effects[49].

The aim of studying polymorphism is to explain the risk of common disease because of its abundance in human genome and high frequency in human population. The cellular effects are due to alteration in gene transcription, post translational or post transcriptional activity or change in the structure of gene product[29].

In coding or noncoding regions of a specific gene, there may be either a single base pair substitution of one nucleotide (SNPs) for another or a variable number of repeats of a short repetitive DNA sequence (VNTR). These variations may influence the rate of gene transcription, the stability of the messenger RNA or the quantity and activity of the resulting protein.

Significance behind SNPs:

Average occurrence of SNPs in DNA is 1/300 nucleotides. They act as biological markers to locate the candidate gene involved in the disease. By means of SNPs we can trace the disease that run through the families.

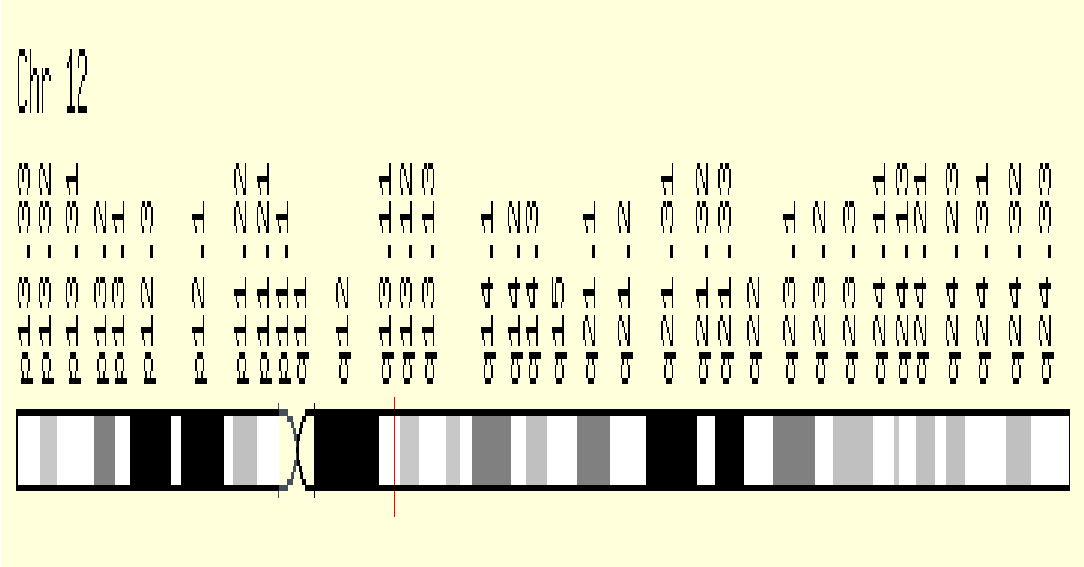
VDR GENE: (rs 731236)

VDR is encoded by a large gene located on the chromosome 12q12-14. The gene is comprised of eleven exons together with the intervening introns spans approximately 75 Kb. The non coding 5'end of gene comprises 3 exons (Type1A, 1B,1C) . Eight exons(2-8)encode the structural portion of gene. VDR gene encompasses two promoter regions, eight protein-coding exons (namely 2-9) and six untranslated exons (1a-1f). It has an extensive promoter region capable of generating multiple tissue-specific transcripts[29].

HGNC approved Gene symbol: VDR

Alternative names	:	1, 25 Dihydroxy vitamin D_3 receptor Vitamin D hormone receptor.
Taxonomy ID	:	9606(NCBI)
Gene ID	:	7421
Common Name	:	Homosapiens(Humans)
Gene Type	:	protein coding
Rank	:	species
Authority	:	Homo sapiens Linnaeus, 1758

Ensembl cytogenic band	**:**	**12q 13.11**
Entrez gene cytogenic band	**:**	**12q 12- q14**
HGNC cytogenic band	**:**	**12q12-q14**
Exon count	**:**	**11**
Annotation release (current)	**:**	**107**
(previous)	**:**	**105**



VDR GENE POLYMORPHISM:

Numerous polymorphisms have been identified in the VDR gene in the recently conducted studies. VDR gene polymorphism occur biologically in human population with significant differences between races and ethnic groups[9].

More than sixty VDR polymorphisms have been discovered till date and they are present in the promoter region, in and around exons and in the 3'UTR (untranslated region). This would affect the expression of gene. Any change in 5' promoter region of VDR gene affect expression of m-RNA, change in exons affect the protein sequence, while 3'UTR affect the stability of m-RNA and efficiency of translation of proteins.

On enzyme digestion DNA fragments of various length are produced and they can be detected by electrophoresis. This is called as Restriction Fragment Length Polymorphisms (RFLP) [30].

Allelic variants of VDR gene recognized by restriction enzymes are:

- Apa1 (allele A/a),
- Bsm1(allele B/b)
- Taq1(allele T/t) ,
- Fok 1(allele F/f).

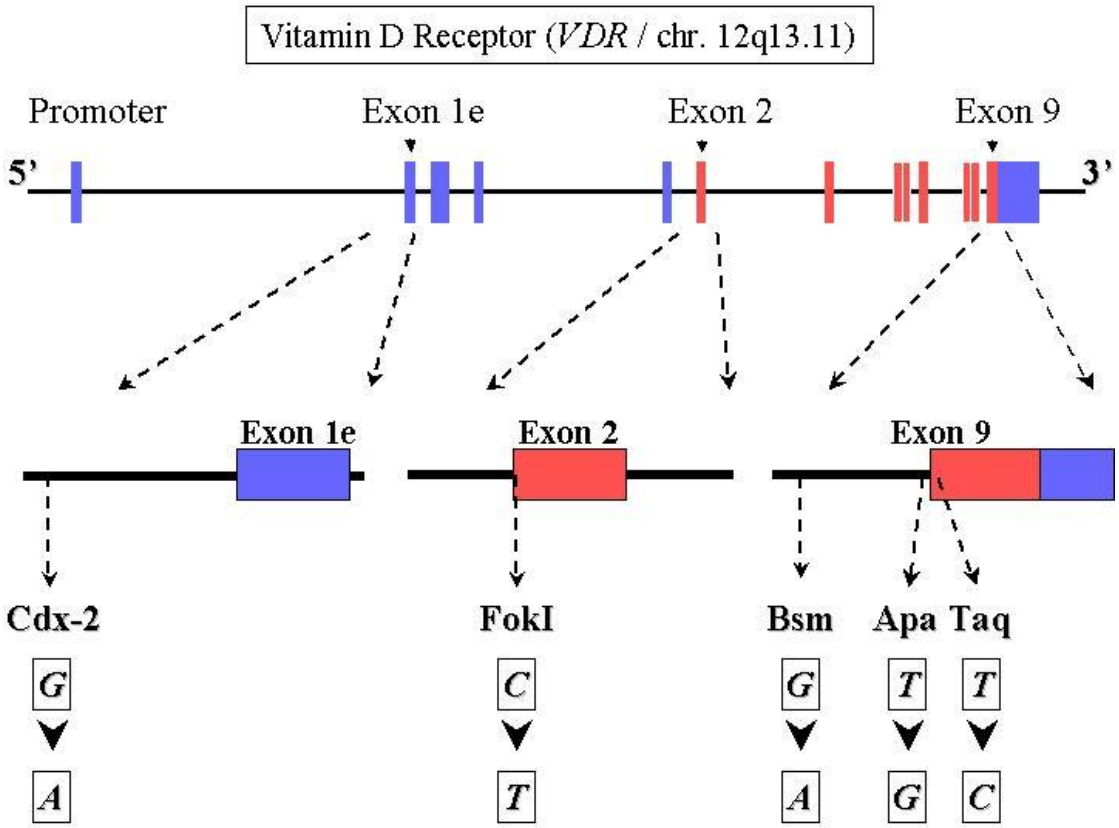
A T/C nucleotide substitution (ATT to ATC) leading to a synonymous change is the Taq1 polymorphism which occurs in exon IX. A T/C transition polymorphism (ATG to ACG) is the Fok1 polymorphism in exon2. BsmI and ApaI are the Restriction site polymorphisms occur in the intron separating exons VIII and IX. The other polymorphism have been reported are Tru91 in

intron VIII, EcoRV located between exons 8 and 9 , Cdx2 in the promoter region and poly(A) mononucleotide repeat in 3' UTR[61].

The association of certain polymorphism with the phenotype does not mean that the polymorphism is causing it. The co-occurrence of alleles of adjacent polymorphisms is called as linkage disequilibrium (LD). In effect the presence of one type of polymorphism can serve as an indication of the presence of another polymorphism linked to it.

A polymorphism in 3' UTR regions of VDR gene appear to be in linkage disequilibrium and the allele frequency vary among the populations. A strong degree of LD has been found among Taq1, BsmI, EcoRV and ApaI RFLPs[30]. LD is also present between Taq1 and Poly (A) since they occur in similar ratios among various ethnic groups. The presence of Taq1 'T' allele in Asians is (8%) compared to Caucasians (43%) and Africans (31%) and similar results for PolyA.

Fig.8 STRUCTURE OF VDR GENE



MATERIALS AND METHODS

AIM OF THE STUDY:

- To determine the frequency of TaqI vitamin D receptor gene polymorphism in normal individuals.
- To determine the frequency of Taq1 vitamin D receptor gene polymorphism in normal individuals and its association with 25-hydroxy vitamin D levels.
- To correlate the values of 25-hydroxy vitamin D levels with the levels of parathyroid hormone, Calcium (Total and Ionized) and Phosphorus.

MATERIALS AND METHODS:

This study was carried out during the period December 2014 - August2015. It is a cross sectional study. The study population comprised of normal individuals coming for master health check up Kilpauk Medical College Hospital, Chennai. All procedures concerning human subjects or patients were permitted by the Institutional Ethical Committee.

STUDY POPULATION:

INCLUSION CRITERIA: Healthy unrelated individuals of age 25-60yrs

EXCLUSION CRITERIA : Persons with diabetes.
Persons with hypertension, Coronary artery disease.
Known alcoholic, smokers etc.

SAMPLE COLLECTION:

For the study, 6 ml of 12 hours Fasting Venous Blood was collected under sterile conditions from the antecubital vein with explicit informed consent.

I. 2ml of blood was collected in plain vials and serum was separated after centrifugation at 3000 rpm for 10 minutes and aliquoted, into an eppendorfs and stored at -20°C and were not thawed until the batch was analyzed for total calcium, phosphorus , total protein and albumin. Before storage ionized calcium is estimated.

II. 2 ml of blood collected in EDTA tube for DNA extraction. Plasma was separated after centrifugation at 3000 rpm for 10 minutes and aliquoted, into an eppendorfs and stored at -20°C and were not thawed until the batch was analyzed for glucose, urea and creatinine.

III. 2ml of blood collected in plain serum tube for analysis of 25-OH vitamin D and PTH ASSAY.

ESTIMATION OF GLUCOSE:

Method: Glucose oxidase peroxidase (GOD/POD) (End Point)
Kit Used*:* Erba

Principle

$$\text{Glucose} + O2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O$$

$$H2O2 + \text{Phenol+4AAP} \xrightarrow{\text{peroxidase}} \text{Quinoneimine dye} + H_20$$

The intensity of pink colored Quinoneimine dye is proportionate to glucose concentration and was measured at 505nm.

Reagent Composition

Reagent 1: Enzyme reagent

Glucose oxidase → ≥ 20000U/L

Peroxidase → ≥ 2000U/L

Phenol → 10 mmol/L

Phosphate buffer → 200 mmol/L

Glucose standard → 100 mg/dl

Procedure

To 1ml of working solution, 10µl of plasma was added and Incubated at 37°c for 15 minutes and absorbance was measured at 505 nm.

Reference range

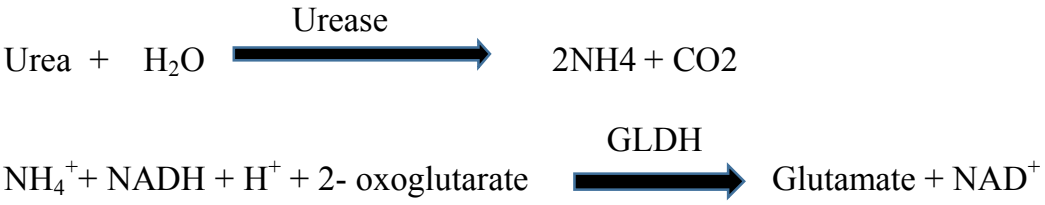
Fasting plasma glucose: 70 –100 mg/dl

ESTIMATION OF BLOOD UREA

KIT : Accucare

Method : UV - GLDH

Principle : The test is performed as a kinetic assay in which the initial rate of the reaction is linear for a limited period of time. Urea is hydrolysed by urease to NH_3 and CO_2. The NH_3 produced combines with alpha-oxoglutarate and NADH in the occurrence of glutamate dehydrogenase to produce glutamate and NAD.



The initial rate of decrease in absorbance is directly proportional to the urea concentration in the sample. Absorbance is measured at 340nm.

Reagent

Reagent I	:	buffer reagent
Reagent II	:	enzyme reagent
Urea standard	:	50 mg/dl

Mix 4 parts (4 ml) of buffer reagent with one part (1 ml) of enzyme reagent and mix gently.

Procedure

To 1 ml of the reconstituted reagent 10µl of plasma is added and absorbance measured immediately at 340 nm.

Reference Range

Serum/ plasma Urea → 15- 40 mg/dl

ESTIMATION OF SERUM CREATININE

Kit used : ERBA

METHOD : Jaffe's Method , Initial rate method

Principle :

Creatinine in alkaline solution reacts with picrate to form a orange-yellow compound. The color is proportional to the concentration of creatinine in the sample when measured at 505nm.

Reagent composition:

Reagent I : Picric acid reagent.

Picric acid – 25.8 mmol/L

Reagent II: Sodium hydroxide reagent.

Sodium hydroxide – 95 mmol/L

Creatinine standard: 2 mg/dl

Reagents were allowed to attain room temperature. Equal volumes of reagent 1 and reagent 2 were mixed , waited for 15 minutes before use .

Procedure

To 1 ml of the reconstituted reagent 100µl of the plasma was added and absorbance (A1) at 20 seconds after mixing was noted & final absorbance ((A2) at 80 seconds were measured.

Calculation

A = A2 - A1

$$\text{Creatinine (mg/dl)} = \frac{\text{Absorbance of Test X concentration of standard (mg/dl)}}{\text{A of standard}}$$

Reference Range:

Males : 0.7 - 1.4 mg/dl

Females : 0.6 - 1.2mg/dl

ESTIMATION OF SERUM CALCIUM:

KIT: SPINREACT

METHOD: ArsenazoIII.

PRINCIPLE: Calcium with ArsenazoIII (1,8–DiHydroxy-3,6-disulpho-2,7naphthalene-bis(azo)-dibenzearsonic acid), at neutral pH, yields a blue colored complex. The intensity of the color is directly proportional to the calcium concentration in the sample.

REAGENTS:

R - IMIDAZOLE BUFFER PH 6.5 → 100mmol/L

Arsenazo III → 120mmol/L

Standard - 10 mg/dl

PROCEDURE:

To 1 ml of the reagent 10µl of serum added. Mix and incubate for 2 min at 37˚C and reading was taken at 650 nm.

REFERENCE RANGE:

Serum/ plasma: 8.5-10.5mg/dl

ESTIMATION OF PHOSPHORUS:

KIT: SPINREACT

METHOD: PHOSPHOMOLYBDATE.UV

PRINCIPLE:

Inorganic phosphate reacts in acid medium with ammonium molybdate to form a phosphomolybdate complex with yellow color. The intensity of the

color is directly proportional to the inorganic phosphorus concentration in the sample.

REAGENTS:

R - **A**mmonium molybdate -→ 0.40 mM

Sulphuric acid → 210Mm

Detergents.

Standard - 5mg/dl

PROCEDURE: To 1ml of the reagent 10µl of serum added. Mix and incubate for 5 minutes and reading taken at 340 nm.

REFERENCE VALUE:

Serum or Plasma- 2.5-5.0 mg/dl

ESTIMATION OF ALBUMIN:

KIT: PRIETEST

METHOD: Bromocresol green, End point

PRINCIPLE:

Serum albumin in the presence of bromo cresol green at a slightly acid ph produces colour change of the indicator from yellow-green to green blue.

Albumin + BCG ----------→ Albumin-BCG complex.

REAGENT:

Succinate buffer → 75mmol/L

Bromo cresol green→ 0.15mmol/L

Standard: 4.0 g/dl

PROCEDURE:

To 1 ml of the reagent 10µl of serum added. Mix and incubate for 5 minutes and reading taken at 620 nm.

REFERENCE RANGE: 3.5 – 5.2 g / dl.

ESTIMATION OF TOTAL PROTEIN:

KIT: ACCUCARE

METHOD: BIURET , End point

PRINCIPLE:

Proteins give intensive violet blue colored complex with copper salt in a alkaline medium, iodide is included as a antioxidant . The intensity of the color is directly proportional to the concentration of protein in the sample.

REAGENT : Biuret reagent

Standard: 6g/dl

PROCEDURE:

To 1 ml of the reagent 20µl of serum added. Mix and incubate for 10 minutes and reading taken at 540 nm.

REFERENCE RANGE:

Serum/ plasma: 6.6 to 8.3 g/dl.

ESTIMATION OF IONIZED CALCIUM:

Serum ionized calcium measured by Ion selective electrodes by Eletrolyte analyser 9180 (ROCHE)

Reference range: 1.15 to 1.33mmol/dl

ESTIMATION OF 25-OH VITAMIN D LEVELS:

25-OH vitamin D in serum is determined using ADVIA CENTAUR vitamin D assay on ADVIA CENTAUR XP systems.

METHOD: CLIA

PRINCIPLE:

The ADVIA Centaur VitD assay is an 18-minute antibody competitive immunoassay that uses an anti-fluorescein monoclonal mouse antibody covalently bound to paramagnetic particles (PMP), an anti-25(OH)vitamin D monoclonal mouse antibody labeled with acridinium ester (AE), and a vitamin D analog labeled with fluorescein.

An inverse relationship exists between the amount of vitamin D present in the patient sample and the amount of relative light units (RLUs) detected by the system.

REFERENCE RANGE:

VIT D: 10 – 30 ng/ml

ESTIMATION OF INTACT PTH ASSAY:

Determination of intact parathyroid hormone in serum using ADVIA CENTAUR i PTH assay on ADVIA CENTAUR XP systems.

METHOD: CLIA

PRINCIPLE:

The ADVIA Centaur Intact PTH assay is a two-site sandwich immunoassay uses direct chemiluminometric technology. It uses constant

amounts of two anti-human PTH antibodies in the Lite Reagent. The polyclonal goat anti-human PTH (N-terminal 1-34) antibody labeled with acridinium ester is the first antibody. The biotinylated polyclonal goat anti-human PTH (39-84 regions) antibody is the second antibody. Streptavidin in the solid phase is covalently coupled to paramagnetic latex particles.

REFERENCE RANGE: 11.1 -79.5pg/ml

For gene polymorphism studies fasting blood was collected in 2 ml EDTA coated tube.

I. DNA EXTRACTION BY KIT METHOD:

The Mag pure human blood genomic DNA Preparation kit: From Helini Biomolecules, Chennai.

PRINCIPLE:

RBCs and cells are incubated with proteinase K in the presence of chatropic salt, which immediately inactivates all nucleases. Following addition of magnetic beads and binding buffer , precipitated cellular nucleic acids selectively bind to magnetic beads. Bound nucleic acid is purified in a series of rapid wash steps to remove contaminating cellular components. Finally low salt elution releases the nucleic acids from the magnetic beads. This sample method eliminates the need for organic solvent extractions, allowing for rapid purification of many samples simultaneously.

COMPONENTS OF THE KIT:

- Proteinase K
- Lysis Buffer
- Binding Buffer
- Wash Buffer-1
- Wash Buffer-2
- Elution Buffer
- Magnetic beads

Proteinase K stored at -20˚C

Mag Pure Magnetic Beads at 4˚C

Buffers at room temperature.

PROCEDURE FOR DNA EXTRACTION:

PRE-PROCEDURE STEPS:

- Set water bath at 70˚C
- Label five 2ml micro centrifuge tube as Lysis, wash-1, wash-2, wash-2 and elute.
- 300µL of lysis buffer is added to the tube labeled as lysis and close the lid.
- 500µL of wash buffer-1 and wash buffer-2 added to appropriate labeled tubes and close the lid.
- 100µL of elution buffer is added to the tube labeled as elute and close the lid.

PROCEDURE:

- 0.2ml of fresh human blood or 0.1 ml of buffy coat into 2ml centrifuge tube labeled as lysis. Mixed well and vortexing done.

1. 20µL of Proteinase K is added and mix well by vortexing, incubated at room temperature for 3 minutes.

2. 40µl of Mag Pure Beads is added and 500µl of binding buffer supplemented with ethanol is added and mixed by vortexing.

3. Using Mag stick with fresh tip MagPure beads are captured and transfer to wash buffer-1 and vortexed briefly.

4. Mag stick with tip is inserted to capture all the beads from wash buffer -1 and transfer to wash buffer-2. Mixed gently by vortexing

5. Magstick with tip to capture all beads and transferred to wash buffer-2. mix gently by vortexing.

6. Mag stick with tip is inserted to capture all beads and transfer to elute. Discard the tip.

7. Resuspend the magnetic beads by brief vortexing and incubated in water bath at 70˚C for 5 minutes.

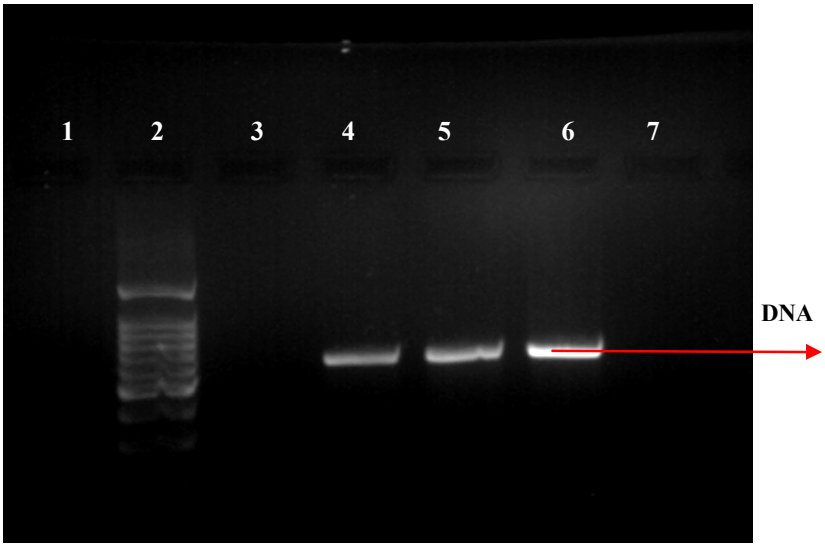
8. Mag stick with fresh tip is inserted to capture Mag pure beads from the elution buffer and the tip is discarded.

9. The elute is transferred into fresh 1.5 ml tube and the purified DNA is used immediately or stored at-20˚C

IDENTIFICATION

Extracted DNA was identified by 1% agarose gel electrophoresis and comparison with a known molecular weight 1kb DNA (Lambda DNA)ladder as depicted in

FIG : 9 EXTRACTED DNA



PCR(polymerase chain reaction) was carried out in a reaction in volume of 50µL with the following components in the following manner:

Red Dye PCR master mix – 25µL

Red Dye PCR master mix – 25µL

Primer mix – 10.0 µL

DNA– 5.0µL

Distilled water- 10µl

Total volume: 50µl

POLYMERASE CHAIN REACTION (PCR)

Amplification of the extracted DNA was carried out in CYBERLAB SMART PCR-PRO, thermal cycler with the following cycling conditions.

STEPS IN PCR TAQ1 POLYMORPHIC SITE:

- Initial denaturation - 95° C /4min

  34cycles of

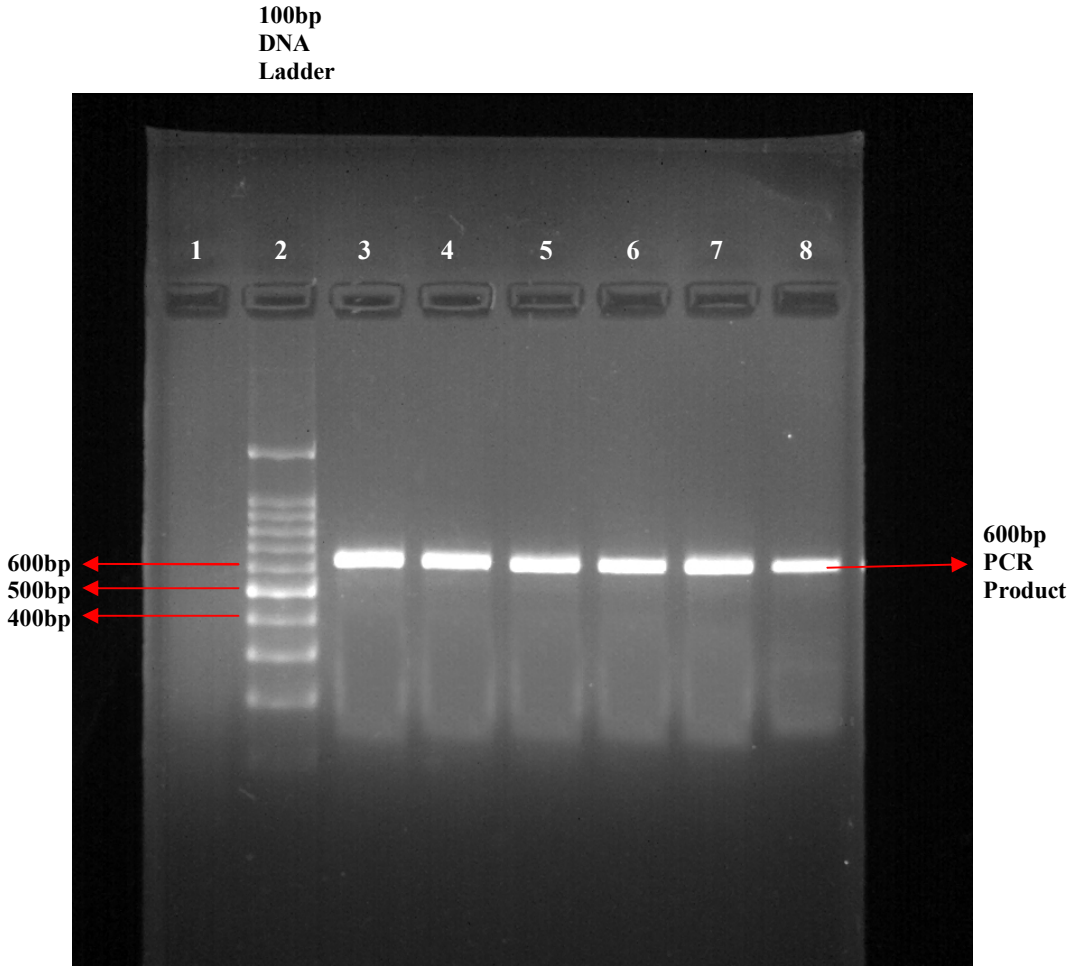
  - Cycle Denaturation at 95°C for 30 seconds
  - Cycle Annealing at 58°C for 30 seconds
  - Cycle Extension at 72°C for 45 seconds
  - Final Extension at 72°C for 5min

Amplified product – amplicons of 600 bp was identified by 2.5% agarose gel electrophoresis by comparison with a known 100bp DNA ladder

AGAROSE GEL ELECTROPHORESIS

- PCR product is run on 2.5% agarose gel in a 25 mL agarose cast as follows: 0.625g of agarose is weighed and dissolved in 25 mL of TAE buffer with a pH of 8.0.
- It was microwaved for 60 secs, cooled and ethidium bromide 0.5 µg/ml was added. It is poured into a cast and allowed to solidify for 45 min before it is kept in the electrophoresis tank.
- 10µL of PCR product is loaded onto wells and 4µL of 100bp DNA ladder is loaded onto single well as a marker. It is run in electrophoresis tank for 30min and visualized under UV illumination in e gel imager from life technologies .

FIG :10 PCR PRODUCT- rs731236



RESTRICTION DIGESTION OF THE PCR PRODUCTS TAQ1 SITE

RESTRICTION FRAGMENT LEGTH POLYMORPHISM

Taq1gene polymorphism at the site was spotted by digestion of the amplified PCR product with the TaqI restriction enzyme trailed by run in 2.5% agarose gel electrophoresis.

Restriction Digestion Procedure

Taq1 buffer	–	5 µL
Taq I enzyme	–	2.5 µL
PCR Product	–	30.0 µL
Distilled water	–	12.5 µL
Total		50.0 µL

The entire procedure is carried out in ice. The contents are mixed thoroughly.

- The eppendorf is then placed in a 65°C waterbath for 30minutes
- Restriction digested product is subjected to 2.5% agarosegel electrophoresis for genotyping.

Two restriction sites for Taq I with in 600 bp

t ALLELE- have restriction site

T ALLELE- absence of restriction site

- **tt (homozygous individuals)**- will yield 400bp,200bp
- **Tt (heterozygous individuals)**- will yield 600, 400bp,200bp

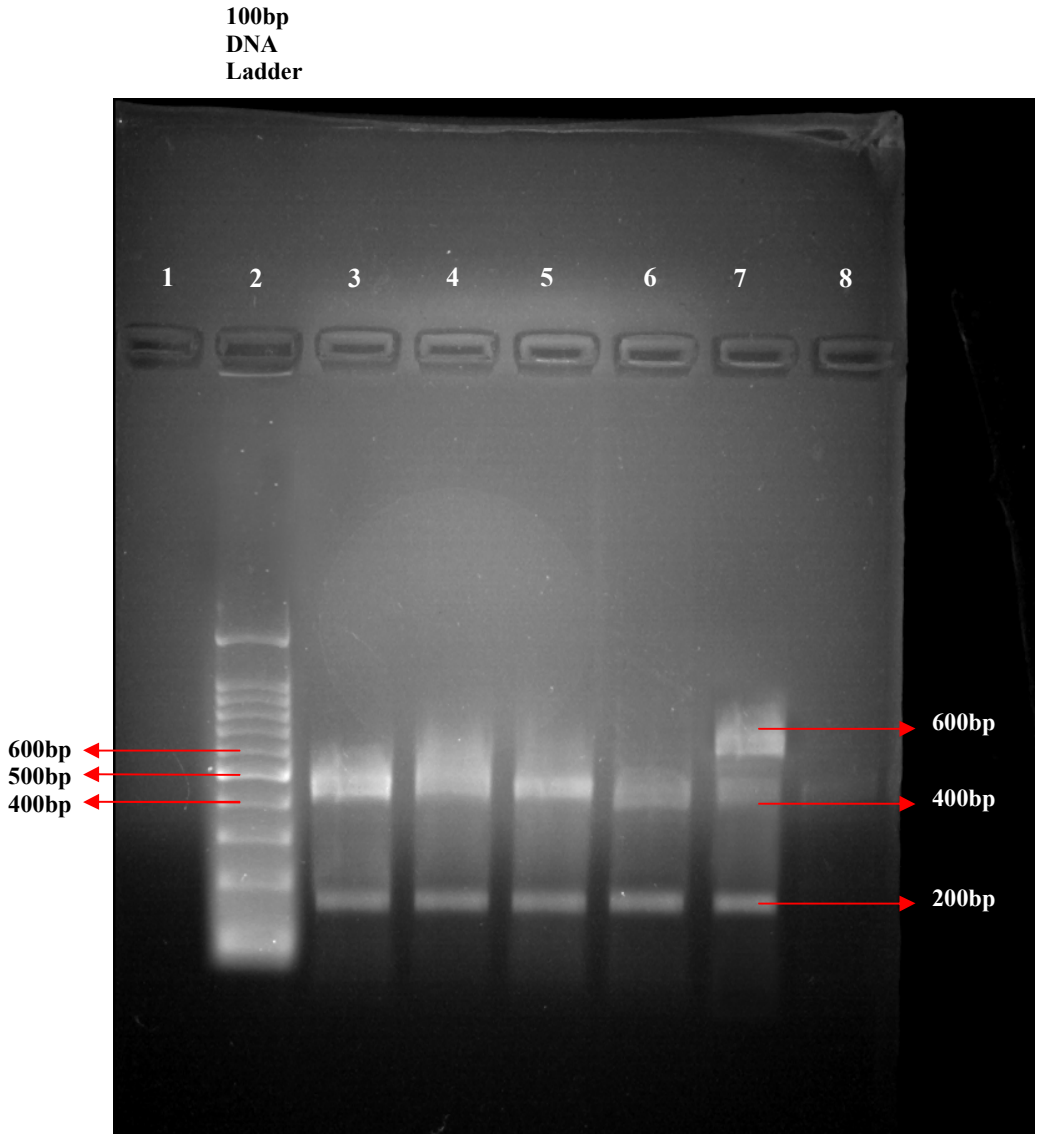
- **TT(homozygous individuals)**- will yield 600bp

RESTRICTION DIGESTED PRODUCT VIEWED BY RUNNING IN 2.5% AGAROSE GEL ELECTROPHORESIS

PROCEDURE:

- Restriction Digestion product is run on 2.5% agarose gel in a 25mL agarose cast as follows: 0.625g of agarose is weighed and dissolved in 25mL of TAE buffer with a pH of 8.0.
- It is microwaved for 60 secs, cooled and Ethidium bromide concentration 0.5 µg/ml from the stock of 10mg/mL is added. It is poured into a cast and allowed to solidify for 45 min before it is kept in the electrophoresis tank.
- 20µL of Restriction Digestion product is loaded onto wells and 4µL of 100bp DNA ladder is loaded onto single well as a marker. It is run in electrophoresis tank for 45min and visualized under UV illumination in E gel imager from life technologies (Fig.)Analysis was done using 100 bp and 50 bp ladder

FIG.11 RESTRICTION DIGESTION PRODUCT- rs731236



RESULTS

TABLE-1 Shows age, glucose, urea and creatinine among study population:

Variable	Number	Mean	Std.dev
Age	100	38.2800	10.07154
Glucose	100	62.6000	22.96506
Urea	100	19.8200	6.08090
Creatinine	100	.8300	0.17321

This table shows the routine parameters (glucose, urea, creatinine)within the study population. And these parameters are within normal limits.

Table-2 Distribution of vitamin D levels in the study population.

Vit D	Group-1 (<10ng/ml)	Group-2 (10-20ng/ml)	Group-3 (>20ng/ml)
Numbers(n)	21 (21%)	43 (43%)	36 (36%)
Mean	6.947	14.31	30.392
Std dev	1.914	2.657	8.085

Based on the vitamin D levels the study population are grouped into three groups.

Group-1 --- <10ng/ml (vit D deficiency)

Group-2 ---- 10-20ng/ml (vit D insufficiency)

Group-3 --- >20ng/ml (vit D sufficiency)

Among the study population about 21% of individuals are vitamin D deficient (group- and 43% individuals are vitamin D insufficient(group-2) and 36% of the population are vitamin D sufficient(group-3)

Table-3 Correlation of vitamin D with parathyroid hormone among the study population

	Vit D (ng/ml)	**N**	**Mean**	**Std dev**	**p value**
PTH	<10	21	68.3143	39.5684	0.002***
	10-20	43	50.0070	25.3879	
	>20	36	41.9211	16.9526	

Table- 3 shows correlation between vitamin D levels and parathyroid hormone.

A significant inverse relation was observed between vitamin D and intact parathyroid hormone levels in all the three groups. As vitamin D level decreases there is a proportionate increase in parathormone level and is found to be statistically significant with a p value of 0.002.

FIG.12 CORRELATION OF VIT.D AND PTH AMONG STUDY POPULATION

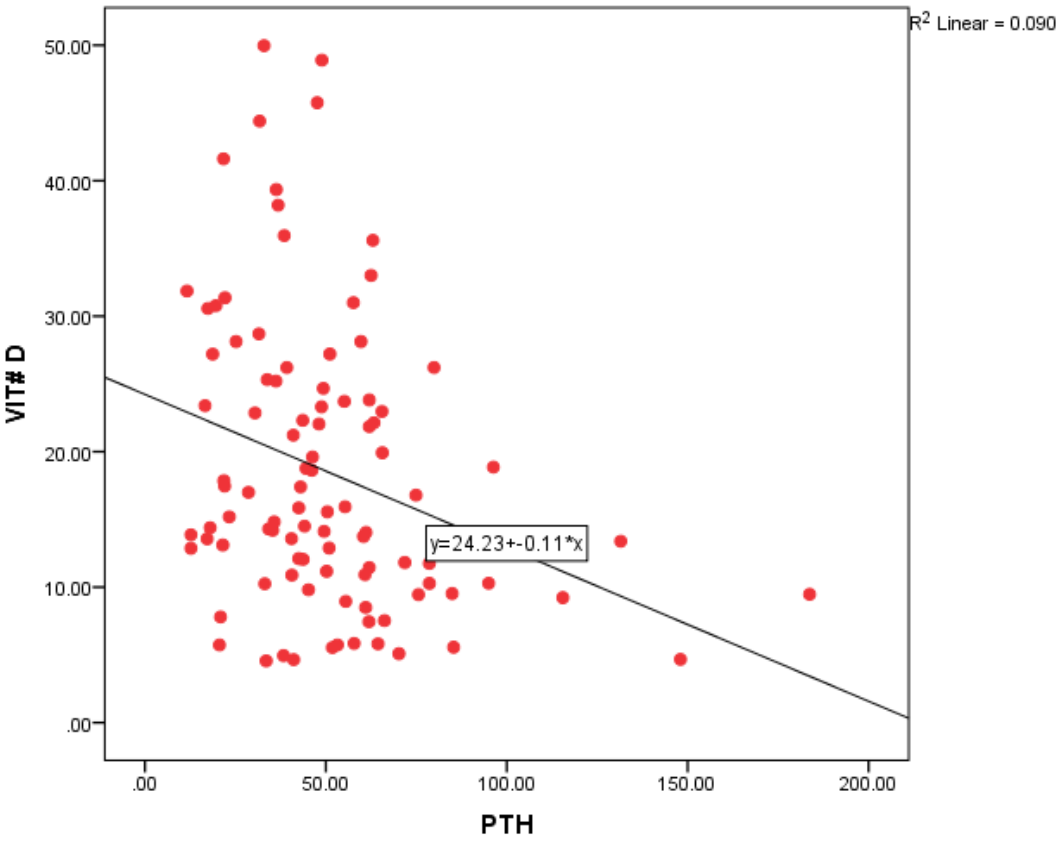


Table -4 Correlation of vitamin D with total calcium, ionized calcium, phosphorus.

	Vit D	N	MEAN	STD DEV	p value
Total calcium	<10	21	8.146	1.03277	< 0.001
	10-20	43	9.2047	1.72157	
	>20	36	11.1778	1.09938	
I.calcium	<10	21	1.1048	0.08664	0.887
	10-20	43	1.1186	0.15001	
	>20	36	1.1056	0.13927	
Phosphorus	<10	21	3.6810	0.80164	0.337
	10-20	43	3.4605	0.57782	
	>20	36	3.4583	0.50787	

The total calcium In group 1 ----7.2- 9.2 mg/dl

In group 2 --- 7.5 – 10.9 mg/dl

In group 3 --- 10.1 -12.1mg/dl

In group 1 the total calcium level is decreased which is statistically significant with a p value <0.001. In group-2 and group-3 the total calcium is within normal limits and it is statistically insignificant.

The ionized calcium and phosphorus are found to be in normal range in all the three groups and it is also statistically insignificant with the p value 0.887 and 0.337 respectively.

.

FIG.13 CORRELATION OF VIT D AND TOTAL CALCIUM

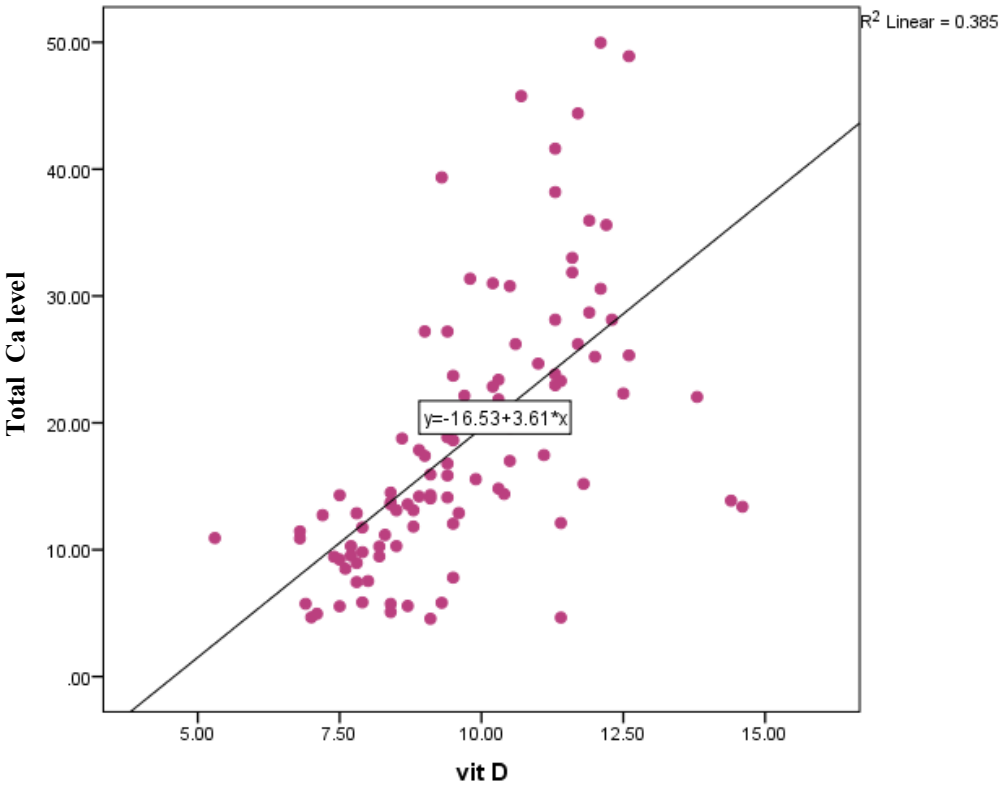


Table -5 Genotype frequency distribution of taq1 gene in study population

Genotype	Frequency	Percent
tt	94	94.0
Tt	6	6.0
Total	100	100.0

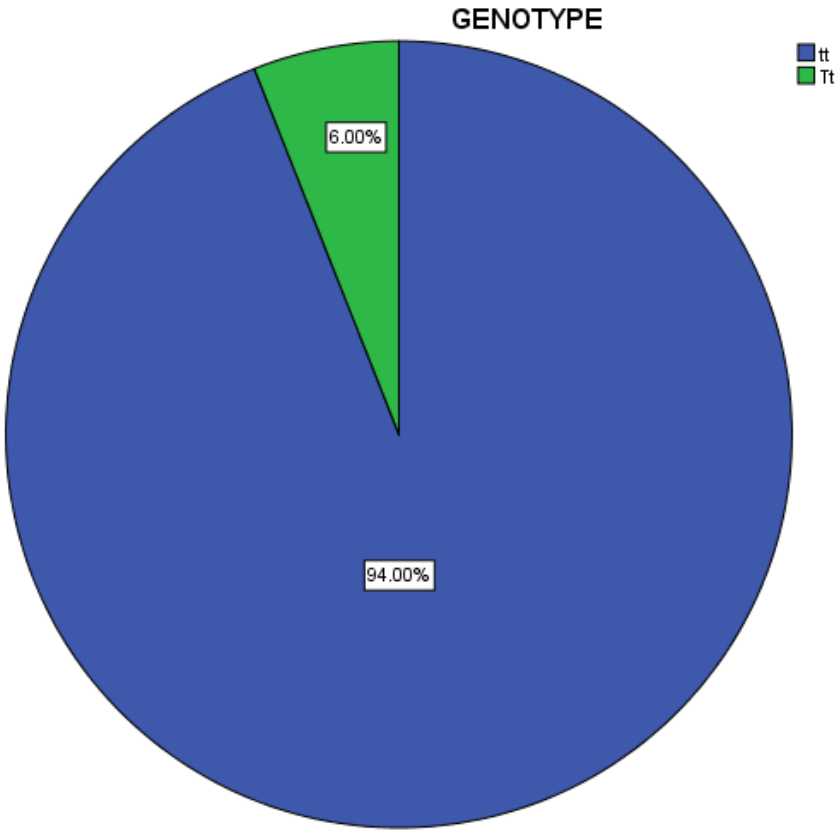
This table shows the genotype distribution among the study population of which the common genotype is homozygous tt genotype which accounts for (94%) and the heterozygous Tt genotype accounts for (6%). There is no homozygous TT genotype in our study population.

Table-6: Correlation between the genotype and vitamin D level in study population:

Genotype	Group1 N (%)	Group2 N (%)	Group3 N (%)	Total N (%)	p value
Tt	21 (22.3%)	39 (41.5%)	34 (36.2%)	94 (100%)	0.49
Tt	0 (0%)	4 (66.7)	2 (33.3%)	6 (100%)	

This shows among the study population, the group-1 individuals have increased frequency of tt genotype of (22.3%) whereas the group-2 individuals have increased frequency of Tt genotype of (66.7%)

FIG.14 PIE CHART SHOWING GENOTYPE DISTRIBUTION



DISCUSSION

The present study is an attempt to evaluate the normal distribution of Taq1 gene polymorphism in unrelated normal healthy individuals in Chennai population.

The genes encoding VDR is located on chromosome 12, and consists of 14 exons which span approximately 75 kilo bases of genomic DNA[67]. In the VDR gene many polymorphisms have been discovered so far and their functional significance and probable effects on disease vulnerability are under trial (zumuda et all).

Of several allelic variants in the VDR gene, one which is present in 3'UTR is Taq1 gene polymorphism. It is a T/C nucleotide substitution (ATT to ATC) leading to a silent change at codon 352 both coding for isoleucine and is in linkage disequilibrium with other variants[28].

This polymorphic site reference ID number is rs731236[48]. Taq1 gene polymorphism is studied in most diseases like cancer, osteoporosis[66], urolithiasis, Type1 DM[64], obesity and coronary artery disease[69].

The possible functional mechanism attributed to polymorphism in 3' UTR include change in VDR gene expression by altering polyadenylation, localization of mRNA, stability of mRNA, and translational efficiency. The stability of mRNA influences gene expression by affecting the amount of mRNA available to be translated and the probable means are:

1. The miRNAs (micro –RNAs)can inhibit both initiation step of translation and steps after that such as premature termination. Many mRNAs have multiple binding sites for miRNA and translation is most efficiently inhibited when multiple miRNAS are bound to the mRNA.
2. Length of 3' UTR is considerable , since 3' UTRs which are longer are associated with decreased levels of gene expression because they contain more miRNA and protein binding sites that are involved translation inhibition.
3. Many 3' UTR has AU rich elements (ARES). Proteins binding to ARES, stability is affected or decay rate of transcripts in a localized manner or affect initiation of translation.
4. 3' UTR contains the sequence AAUAAA which favors several hundred adenine residues called poly (A) tail to be added to the end of mRNA transcript. Poly (A) binding protein (PABP) on binding to this tail contributes regulation of mRNA translation and stability.
5. Some mi RNAs cause suppression of transcription by binding to the complementary sequences in DNA and by attracting the enzymes which directly methylate the DNA[70].

Yug Fang et al identified difference in VDR mRNA expression level and stability between hap1 and hap2 alleles across 3' UTR. The fracture risk hap1 allele causes 15% lower levels of mRNA expression than hap2 in all tested cell lines. This is in line with 30% faster decay or lower stability of VDR mRNA observed in osteoblast cell line[74].

In view of this, a population based study was performed in Chennai population to address the following questions:

1. To find the prevalence of vitamin D deficiency among the study population.
2. To find the frequency of VDR Taq1genotype distribution in the study population.
3. Is VDR gene polymorphism is the cause for differences in the vitamin D status among the study population?

Among the study population about 21% are found to be vitamin D deficient(<10ng/ml), 43% are vitamin D insufficient(10-20ng/ml) and 36% are vitamin D sufficient(>20ng/ml)

Correlation between vitamin D levels and parathyroid hormone, total calcium and ionized calcium were done in our study population and the results are as follows:

In this study the vitamin D levels have negative correlation with intact parathormone levels. As vitamin D level decreases there is a proportionate increase in parathormone level.

The total calcium level was decreased in vitamin D deficiency individuals (<10ng/ml), whereas it was within normal range in other two groups.

Zuberi LM Habib A et al stated in their study, calcium level is decreased only in severe vitamin D deficiency but it is normal in moderate and mild vitamin D deficiency which is similar to our study. Mansoor.S.Habib et al described that about 30% of asymptomatic adults with vitamin D deficieny and secondary hyperparathyroidism have normal levels of calcium [83].

Ionized calcium was within normal range in all the individuals of study population. In vitamin D deficiency, plasma calcium is maintained at the expense of bone calcium but persistence of deficiency leads to fall in calcium levels and secondary hyperparathyroidism[81]. Singh et all reported that there exist a poor correlation between vitamin d deficiency and ionized calcium, so that vitamin D deficiency cannot be predicted by ionized calcium[84].

Serum phosphorus levels are within normal limits and it does not correlate with vitamin D levels in this study similar to the study conducted in Karachi.(Shaheen et al,2012) revealed 30 to 40 % of calcium absorption in intestine is increased by vitamin D and phosphorus absorption by 80%. Vitamin D deficiency leads to secondary hyperparathyroidism which results in loss of phosphorus in urine and decrease the intestinal absorption of phosphorus. This results in low or low normal phosphorus concentration[85].

The second hypothesis of our study about the distribution of genotype is as follows: the distribution of tt genotype is significantly higher among the study population. In this study population, we found 94% of tt genotype, 6%

of Tt genotype and the homozygous TT genotype was not found The presence ofTaq1 T allele in Asians is (8%) compared to Caucasians (43%) and Africans (31%) as stated in Kim Kostner et al[48].

In this study the frequency of T allele is 6%. The finding of this study is similar to a study done in Austria (Ewald et al) where the frequency of tt genotype was found higher than TT genotype[8]

The genotype distribution in this study is not similar compared to the studies done so far in Japan (Tokita et al,1996), in china(kung et al 1996), American black Pennsylvania (zumuda et al, 1997), North India (Hemant K Bid et al 2005)

The genotype distribution was not found to be in agreement with Hardy Weinberg equilibrium in our study. Zumuda et al in his study describes that distribution of VDR genotypes was not in Hardy Weinberg equilibrium, the reason behind this may be, apart from genotyping errors, there may be fluctuations due to small samples, nonrandom mating, and migration into or out of the population, selective survivorship among genotypes, population stratification, and admixture of different ethnic groups[67].

Hemant k bid et al in their studies says that there was a lack of data regarding the allelic variations in taq1 genotype from Indian subcontinent where population ethnicities are quite common[6].

The third hypothesis which focuses influence of polymorphism on the vitamin D status showed that Taq1 polymorphism does not influence vitamin D status.

Further attempt should be mandatory in order to understand the molecular and cellular variations affected by the polymorphism and to execute observational studies in larger populations. Study of different haplotypes, instead of SNPs could reduce the inconsistencies found so far, until then the role of VDR polymorphism will still be a topic for debate.

CONCLUSION

- From this study we conclude that the predominant VDR Taq1 genotype in Chennai population is tt genotype. .
- Also the homozygous genotype (TT) was not obtained in our study population.
- About 21% of individuals are vitamin D deficient.
- When genotypes are analysed with respect to 25(OH)D ,no correlation was observed with Taq1 SNP.

LIMITATIONS

Limitations of this study are:

- Small sample size.
- Probable occurrence of linkage disequilibrium of Taq1 gene with other genes like Bsm1, Apa1 in the 3' UTR.
- Difference in race, diet, or latitude may modify the influence of polymorphisms on propensity of disease, diluting the effects observed in other population.
- Study of different haplotypes instead of SNPs would help in clarifying the molecular mechanism underlying the associations observed in 3'UTR polymorphisms

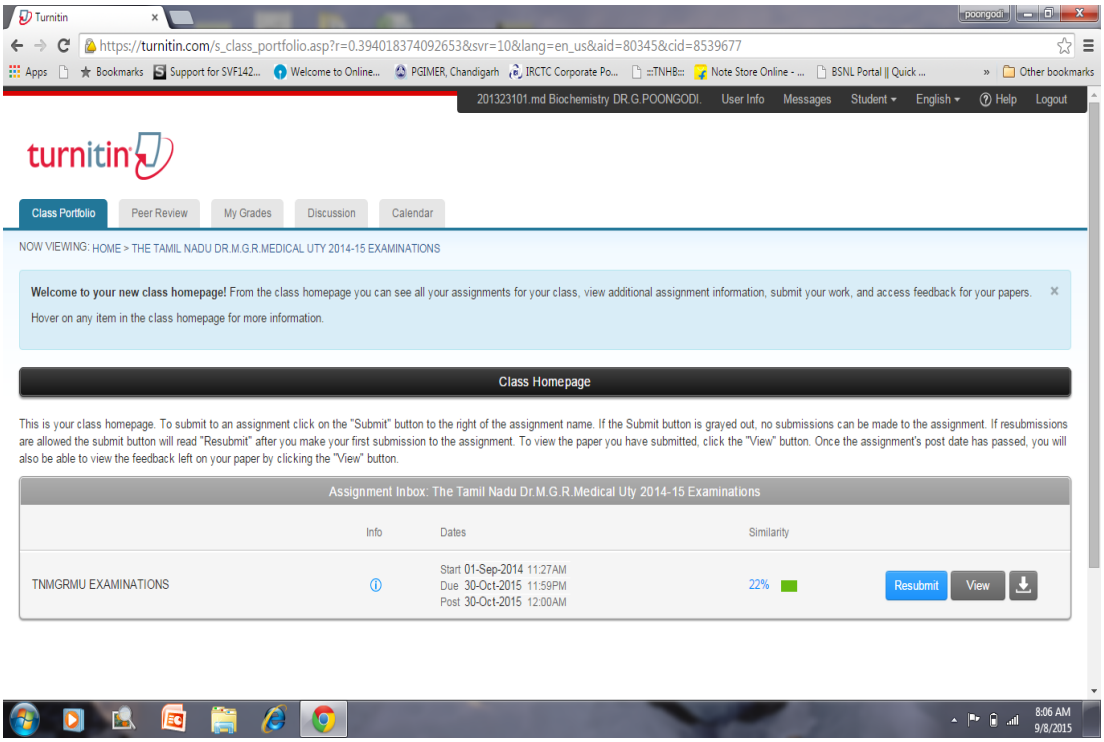
SCOPE FOR FURTHER STUDY

- More studies with large samples are needed to confirm the genotype distribution in the study population.

- Probable occurrence of linkage disequilibrium of Taq1 gene with other VDR genes like APA1, BSM1 in 3' UTR site has to be established.

- Whole genome sequence analysis is needed to reveal extensive level of variation and heterogeneity between individuals and populations and genome-wide association studies (GWAS) has to be done , as these analysis eliminates bias in the selection of the candidate genes.

ANNEXURE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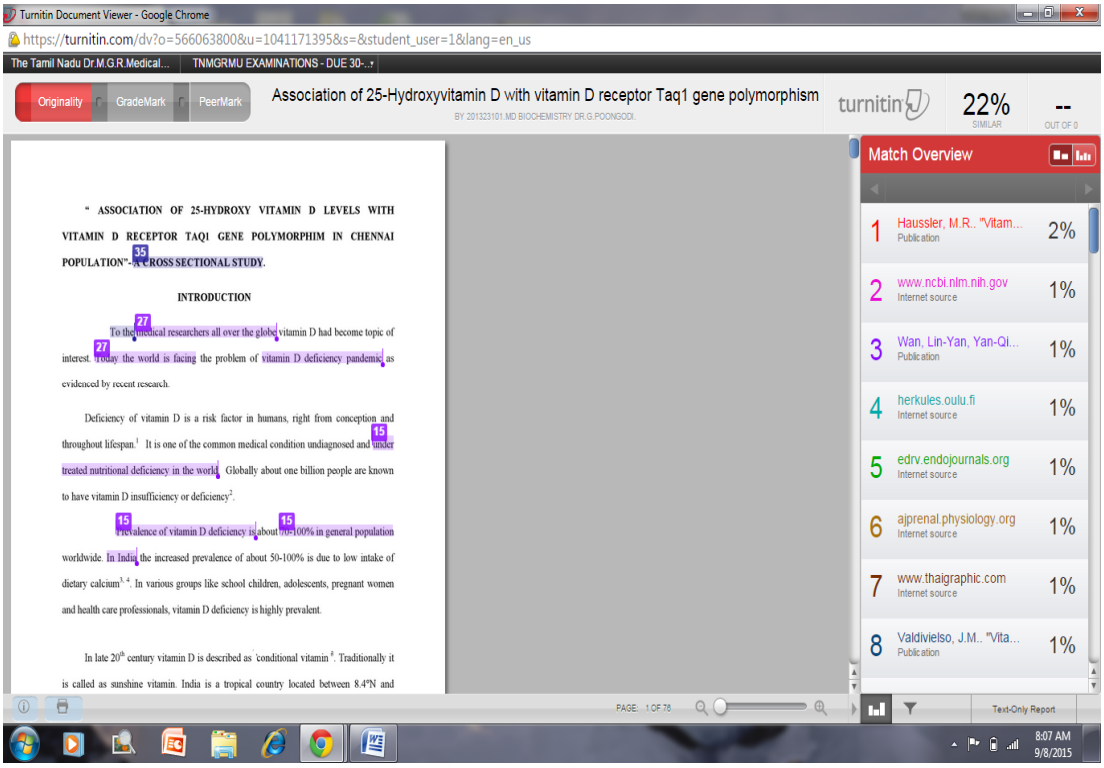
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" ASSOCIATION OF 25-HYDROXY VITAMIN D LEVELS WITH VITAMIN D RECEPTOR TAQI GENE POLYMORPHIM IN CHENNAI POPULATION"- A CROSS SECTIONAL STUDY.
INTRODUCTION
To the medical researchers all over the globe vitamin D had become topic of interest. Today the world is facing the problem of vitamin D deficiency pandemic as evidenced by recent research.
Deficiency of vitamin D is a risk factor in humans, right from conception and throughout lifespan.[1] It is one of the common medical condition undiagnosed and under treated nutritional deficiency in the world. Globally about one billion people are known to have vitamin D insufficiency or deficiency[2].
Prevalence of vitamin D deficiency is about 50-100% in general population worldwide. In India the increased prevalence of about 50-100% is due to low intake of dietary calcium[3, 4]. In various groups like school children, adolescents, pregnant women and health care professionals, vitamin D deficiency is highly prevalent.
In late 20th century vitamin D is described as 'conditional vitamin'. Traditionally it is called as sunshine vitamin. India is a tropical country located between 8.4°N and
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ABBREVIATIONS:

VDD	–	Vitamin D Deficiency
VDR	–	Vitamin D Receptor
VDRE	–	Vitamin D Response Element
RFLP	–	Restriction Fragment Length Polymorphism
25-(OH) D	–	25-Hydroxy Vitamin D
1, 25$(OH)_2$ D	–	1,25 Di Hydroxy Vitamin D
PTH	–	Parathyroid Hormone
DBP	–	Vitamin D Binding Protein
SZA	–	Solar Zenith Angle
TRPV	-	Transient Receptor Potential Vanniloid
OPG	–	Osteoprotegrin
DM	–	Diabetes mellitus
RXR	–	Retinoid X Receptor
VDIR	–	VDR Interacting Repressor
CTE	–	C -Terminal Extension

DBD	–	DNA Binding Domain
LBD	-	Ligand Binding Domain
nVDRE	–	Negative vitamin D response Elements
HAT	–	Histone Acetyl Transferase
SRC	–	Steroid Receptor Coactivator
SNPs	–	Single Nucleotide Polymorphisms
UTR	–	Un translated Region
miRNAs	–	Micro RNAs
PCR	–	Polymerase Chain Reaction
LD	–	Linkage Disequilibrium

INSTITUTIONAL ETHICAL COMMITTEE
GOVT.KILPAUK MEDICAL COLLEGE,
CHENNAI-10

Protocol ID No.05/01/2015 Dt.20. 01.2015

CERTIFICATE OF APPROVAL

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval "Association of 25- Hydroxy Vitamin D with Vitamin D Receptor Taq1 Gene Polymorphism in Chennai Population."A Cross Sectional Study - For Project Work-submitted by Dr.G.Poongodi, II nd Year MD., Biochemistry, PostGraduate Student, KMC, Chennai-10.

The Proposal is APPROVED.

The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

20/1/15

CHAIRMAN,
Ethical Committee
Govt.Kilpauk Medical College,Chennai

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6 Comp.> ME 1> Ethical Committee

நோயாளி ஒப்புதல் படிவம்

ஆராய்ச்சியின் விவரம்: சாதாரண மக்களுக்கு உள்ளமரபணுVDR,Taq1பல்லுருத்தோற்றத்தினால் உண்டான தொடர்பு

ஆராய்ச்சி மையம்: அரசு கீழ்ப்பாக்கம் மருத்துவக் கல்லூரி மருத்துவமனை

நோயாளியின் பெயர்: **நோயாளியின் வயது:**

பதிவு எண்: **முகவரி:**

1. மேற்குறிப்பிட்டுள்ள ஆராய்ச்சியின் நோக்கத்தையும்; பயனையும் முழுவதுமாக புரிந்துக்கொண்டேன். மேலும் எனது அனைத்து சந்தேகங்களையும் கேட்டு அதற்கான விளக்கங்களையும் தெளிவுபடுத்திக் கொண்டேன்.
2. மேலும் இந்த ஆராய்ச்சிக்கு எனது சொந்த விருப்பத்தின் பேரில் பங்கேற்கிறேன் என்றும்; மேலும் எந்த நேரத்திலும் எவ்வித முன்னறிவிப்புமின்றி இந்த ஆராய்ச்சியிலிருந்து விலக முழுமையான உரிமை உள்ளதையும்; இதற்கு எவ்வித சட்ட பிணைப்பும் இல்லை என்பதையும் அறிவேன்.
3. ஆராய்ச்சியாளரோ; ஆராய்ச்சி உதவியாளரோ; ஆராய்ச்சி உபயத்தாரோ; ஆராய்ச்சி பேராசிரியரோ; ஒழுங்கு நெறி செயற்குழு உறுப்பினர்களோ எப்போது வேண்டுமானாலும் எனது அனுமதியின்றி எனது உள் நோயாளி பதிவுகளை இந்த ஆராய்ச்சிக்காகவோ; அல்லது எதிர்கால பிற ஆராய்ச்சிக்களுக்காகவோ பயன்படுத்திக் கொள்ளலாம் என்றும் மேலும் இந்த நிபந்தனை நான் இவ்வாராய்ச்சியிலிருந்து விலகினாலும் தகும் என்றும் ஒப்புக்கொள்கிறேன். ஆயினும் எனது அடையாளம் சம்பந்தப்பட்ட எந்த பதிவுகளும் (சட்ட பூர்வமான தேவைகள் தவிர) வெளியிடப்படமாட்டாது என்ற உறுதிமொழியின் பெயரில் இந்த ஆராய்ச்சியிலிருந்து கிடைக்கப்பெறும் முடிவுகளை வெளியிட மறுப்பு தெரிவிக்கமாட்டேன் என்று உறுதியளிக்கின்றேன்.
4. இந்த ஆராய்ச்சிக்கு நான் முழுமனதுடன் சம்மதிக்கின்றேன் என்றும் மேலும் ஆராய்ச்சிக் குழுவினர் எனக்கு அளிக்கும் அறிவுரைகளை தவறாது பின்பற்றுவேன் என்றும் உறுதியளிக்கின்றேன்.
5. இந்த ஆராய்ச்சிக்குத் தேவைப்படும் அனைத்து மருத்துவர் பரிசோதனைகளுக்கும் ஒத்துழைப்பு தருவேன் என்று உறுதியளிக்கிறேன்.
6. இந்த ஆராய்ச்சியில நோய்க்கான பரிசோதனைகளுக்கும் மேலும் மரபணு சோதனையும் மேற்கொள்ளப்படுகிறது என்பதை ஆராய்ச்சியாளர்கள் மூலம் அறிந்து கொண்டேன். மரபணு சோதனைக்கும் எனது முழு ஒப்புதலை தருகிறேன்.
7. இந்த ஆராய்ச்சிக்கு யாருடைய வற்புறுத்தலுமின்றி எனது சொந்த விருப்பத்தின் பேரிலும் சுய அறிவுடனும் முழுமனதுடனும் சம்மதிக்கின்றேன் என்று இதன் மூலம் ஒப்புக்கொள்கிறேன்.

நோயாளியின் கையொப்பம் / பெருவிரல் கைரேகை

இடம்: தேதி:

ஆராய்ச்சியாளர் கையொப்பம்:

இடம்: தேதி:

PROFORMA

Date:

Name: **OP/IP No:** **SAMPLE ID:**

Age: **Sex :**

Address: **Occupation:** **State:**

PRESENTING COMPLAINTS:

PAST HISTORY:

Liver disease/Kidney disease/ DM/ Skeletal disease/ Drugs/

TREATMENT HISTORY:

PREVIOUS HOSPITALIZATION:

PERSONAL HISTORY: Smoking/ Alcohol intake.

O/E:

Built Height- Weight-

BMI- Waist-Hip-Ratio

Pedal edema/Anemia/Clubbing /Lymphadenopathy

VITALS: BP: PulseRate:

SYSTEMIC EXAMINATION :

CVS: Abdomen :

RS : CNS:

Diagnosis:

Investigations:

1. Blood glucose,urea,creatine
2. 25- HydroxyVitamin D levels
3. Intact Parathyroid hormone
4. Serum Phosphorus
5. Serum Calcium(Total &Ionized)
5. Serum Protein
6. Serum Albumin
7. Genotype:TT/Tt/tt

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S.No.	NAME	AGE	GENDER	GLUCOSE	UREA	CREATININE	T. CALCIUM	ION. CALCIUM	PHOSPHORUS	T.PROTEIN	ALBUMIN	VIT. D	PTH	GENOTYPE
1	LILLYMARY	42	F	72	13	0.8	8.4	1.2	3.4	8.4	3.7	5.73	20.6	tt
2	AKILABANU	27	F	72	15	0.8	9.1	1.3	3.6	6.9	3.1	4.56	33.5	tt
3	ANANDHI	25	F	56	20	0.8	7.7	1.2	4	7	4	9.53	84.9	tt
4	ILAYARAJA	26	M	44	18	0.9	9.5	1.1	2.8	7.1	4.6	12.05	43.7	tt
5	EZHIL	32	F	83	10	0.8	7.9	1.1	3.4	7.2	3.9	5.85	57.8	tt
6	GEETHA	42	F	58	19	0.8	11.1	1.2	3.1	6.7	3.9	17.46	22	tt
7	AJESH	25	M	63	16	1.1	10.6	1.2	3.5	7.9	4.3	26.21	39.2	tt
8	KALAIARASI	27	F	62	20	0.6	11.4	1.1	3.5	6	3.3	23.31	48.8	tt
9	HARIKRISHNAN	44	M	38	10	1	11.7	0.7	2.5	5.9	3.1	26.21	79.9	tt
10	NOORJAHAN	25	F	62	14	0.8	9.4	1.2	3.8	6.4	3.7	18.86	96.3	Tt
11	VANAJA	33	F	40	15	0.6	12.3	1.2	3.1	6.4	3.7	28.13	59.7	tt
12	POONGODI	34	F	76	22	1.1	12.1	1.2	3.1	7.6	3.6	30.57	17.4	tt
13	MOHANRAJ	39	M	64	15	0.9	9.4	1.1	3.5	5	3.5	15.85	42.5	tt
14	SARAVANAN	40	M	69	22	1.2	6.9	1.1	3.4	6.8	3.8	5.74	53.2	tt
15	UDAYA	36	F	55	16	0.7	7.5	1.2	3.3	7.9	3.9	9.23	116	tt
16	ABZAL	39	M	149	18	1	7.6	1.1	3.6	7.5	4.5	8.5	61	tt
17	MOWRI	34	M	56	25	1.2	5.3	0.9	4.3	7.7	4.9	10.92	60.8	tt
18	LATCHIYA	32	M	40	16	1.1	7.8	1.1	2.8	6.9	4.2	12.87	12.7	tt
19	JAYABHARATHI	42	F	73	22	0.8	10.5	1.2	4.3	6.3	4	17	28.6	tt
20	BHOOMATHI	26	F	62	14	0.7	7	1.1	2.6	5.7	4	4.67	148	tt
21	VEL	29	M	43	18	0.9	8.4	1.1	3.4	6.4	3.6	13.57	17.2	tt
22	JAGAN	26	M	40	22	1.1	7.1	1	4.4	7.9	4.9	4.95	38.3	tt
23	GANGA	43	F	62	22	0.9	8.8	1.1	3.1	4.6	3.3	13.11	83.5	tt
24	SUMATHI	42	F	80	19	0.8	9.1	1.1	3.4	7.2	4	14.31	34.8	tt
25	RAMAYEE	40	F	52	17	0.9	6.8	0.9	2.8	6.6	4.3	11.45	62	tt
26	PRABAKAR	40	M	61	25	0.7	11.3	1.1	3.4	6.2	4.2	28.13	25.2	Tt
27	SIVARANJANI	25	F	62	14	0.8	10.5	1.1	2.7	7.1	4.4	19.61	46.3	tt
28	KUMAR	30	M	53	21	1	10.3	1.1	3.9	6.9	3.8	23.4	16.6	tt
29	GOMATHI	40	F	79	31	0.9	9	0.6	3.7	7.1	4.3	17.4	43	tt
30	VENKATRAMAN	40	M	73	36	1.3	9.8	0.5	3.2	7.4	4.4	31.36	22.1	tt
31	THENMOZHI	48	F	78	21	0.8	9.1	1.2	3.9	7.2	3.2	14.04	61.1	tt
32	VIJAYA	53	F	64	13	0.6	11.4	1.1	4.1	7.7	4.3	21.22	41	tt
33	GEETHA	59	F	95	21	0.8	14.6	1.1	3.2	8.1	4.2	13.39	132	tt
34	MUTHUKUMAR	47	M	64	22	0.9	12.5	1	3.3	5.7	3.7	22.31	43.6	tt
35	AMBIGA	53	F	51	18	0.7	8.6	1.2	3.7	7.3	5.1	18.77	44.5	tt

S.No.	NAME	AGE	GENDER	GLUCOSE	UREA	CREATININE	T. CALCIUM	ION. CALCIUM	PHOSPHORUS	T.PROTEIN	ALBUMIN	VIT. D	PTH	GENOTYPE
36	INDIRA	45	F	54	29	0.8	8.4	1.1	3.4	6	3.7	14.5	44.1	tt
37	ARUNDHADHI	52	F	68	21	0.8	13.8	1.1	3.6	5.9	3.4	22.04	48.1	tt
38	DURGA	27	F	60	15	0.7	8.3	1.1	3	8	4.5	11.17	50.2	tt
39	GOPALAKRISHNAN	30	M	67	20	0.9	11.3	1.1	3.5	6.6	4.4	41.62	21.7	tt
40	PONMANI	52	F	64	19	0.7	11.9	1.2	3.5	7	3.7	28.7	31.5	tt
41	DAVID	47	M	75	25	1	12.6	1.1	2.9	7.2	3.5	48.9	48.9	tt
42	SURYA	28	F	39	29	0.8	11.4	1.1	3.2	6.4	3.9	12.11	42.5	tt
43	ANURADHA	44	F	53	14	0.6	9.3	1	3.5	6.1	3.4	5.82	64.4	tt
44	RANI	58	F	59	15	0.6	11.4	1.1	3.3	9.4	4.3	4.65	41.1	tt
45	SELVI	32	F	196	22	0.8	14.4	1.1	3.5	7.6	4.6	13.87	12.7	Tt
46	THILAGA	26	F	70	22	1	9.5	1.1	4.9	6.9	3.3	7.8	20.9	tt
47	KRISHNAVENI	27	F	58	19	0.8	10	1	4	7.2	4.9	19.92	65.6	tt
48	CHELLAKANNU	58	M	66	22	0.8	8.2	0.9	3	7.3	4.9	9.47	184	tt
49	KUMARI	55	F	84	22	0.9	8.5	1.1	3.5	9.4	4.6	13.11	21.5	tt
50	SELVI	29	F	87	33	0.9	9.9	1.2	3.4	6.2	3.9	15.56	50.4	tt
51	ALAMELU	38	F	65	11	0.6	11.8	1.1	3.4	6.4	3	15.19	23.3	tt
52	AROKYA	38	F	57	21	0.8	11.7	1.2	3.3	7.9	3.8	44.4	31.7	tt
53	JAYALAKSHMI	58	F	78	23	1	12.1	1.1	3.5	7.6	3.5	49.97	32.9	tt
54	MALLIGA.B	50	F	59	33	1	9.5	1.2	3.2	6.9	4.2	18.62	46.1	tt
55	SELVARAJ	25	M	38	27	1	9.4	1.2	4.4	9.6	3.8	27.2	18.7	Tt
56	SUSEELA.B	29	F	76	26	0.9	12	1.2	3.4	7.4	3.8	25.21	36.2	tt
57	JAGAN	31	M	62	29	1	12.6	1.1	3.6	6.6	3.8	25.32	33.8	tt
58	MANGAMMA	38	F	64	27	0.9	11.9	1.2	3.7	6.3	3.3	35.95	38.5	tt
59	KOKILA	28	F	80	26	0.7	9.6	1.1	3.2	7.1	3	12.89	50.9	tt
60	BALAJI	28	M	68	32	1.1	11.3	1.1	2.7	5.3	3	23.82	62	tt
61	JOTHI	54	F	85	31	1.2	8.9	1.1	4.1	7.7	4.4	14.2	35.3	Tt
62	VENKATESAN	25	M	59	24	0.6	10.4	1.2	3.4	6.6	4.4	14.39	18	tt
63	SANTHAMARY	55	F	67	32	0.9	11	1.1	3	8.3	4.6	24.67	49.3	tt
64	MARYSUBILA	34	F	47	20	1	12.2	1.1	4.4	8.2	4.5	35.6	63	tt
65	MEERA.V	50	F	56	20	1	10.7	1.1	3.4	7.4	4.4	45.76	47.6	tt
66	SELVI.B	34	F	52	33	0.8	9.4	1.2	3	7.4	4.3	16.79	74.9	tt

S.No.	NAME	AGE	GENDER	GLUCOSE	UREA	CREATININE	T. CALCIUM	ION. CALCIUM	PHOSPHORUS	T.PROTEIN	ALBUMIN	VIT. D	PTH	GENOTYPE
67	SRIMATHY	26	F	48	19	0.8	8.4	1.8	2.7	5.1	3	13.74	60.4	tt
68	MYTHREHI	25	F	43	18	0.7	7.8	1.1	3.5	6.7	4.9	8.95	55.5	tt
69	PANCHAVARNAM	54	F	58	30	0.8	10.2	1	3	6.4	4.3	31	57.6	tt
70	ISAQBASHA	32	M	43	15	0.8	8.7	1.1	2.8	6.7	4.1	13.58	40.5	tt
71	RANI	54	F	64	24	0.7	7.8	1.1	3.7	8.1	5.2	7.45	61.9	tt
72	AMUDHA	45	F	53	12	0.7	7.4	1	2.4	6.5	3.7	9.45	75.6	tt
73	KOKILA.B	42	F	67	15	0.8	10.3	1.2	3.5	7.2	4.5	21.85	62	tt
74	SHYMALA	47	F	55	20	0.9	7.5	1.1	3	8.3	4.2	14.3	34.2	Tt
75	PORKODI	38	F	55	12	0.7	7.9	1.2	3	8	4.5	11.76	78.6	tt
76	KALPANA	36	F	33	16	0.9	7.7	1.1	5	5.4	3.6	10.28	78.6	tt
77	MAHESH	40	M	48	11	0.6	8.4	1.1	5.6	5.9	3.9	5.09	70.2	tt
78	REGINA	25	F	40	13	0.6	10.2	1.2	2.8	7.3	4.6	22.86	30.4	tt
79	AMUDHA.B	37	F	118	10	0.7	11.3	1.2	2.8	9.8	4.3	38.2	36.8	tt
80	LEELAVATHY	50	F	29	15	0.5	8.8	1.2	3.2	8.3	4.3	11.82	71.8	tt
81	KRISHNAVENI	28	F	32	17	0.7	7.9	1.2	4.9	8	4.8	9.81	45.2	tt
82	SHREELAKSHMI	25	F	45	11	0.5	6.8	1.1	4.9	7.3	4.2	10.89	40.6	tt
83	KANMANI	48	F	67	13	0.7	10.3	1.1	3.6	6.7	4.4	14.82	35.7	tt
84	DHIVIYA	26	F	30	17	0.8	9.1	1.1	4.3	7.1	3.8	15.93	55.3	tt
85	USHA	45	F	76	20	0.9	8	1.1	4.4	6.1	4.2	7.53	66.2	tt
86	VIMALI	29	F	86	19	1	8.5	1.1	4.3	6.2	3.4	10.29	95	tt
87	AROKYA	43	F	67	24	0.5	8.7	1.1	2.6	6.5	3.8	5.57	85.3	tt
88	RAMANI	43	F	78	15	0.7	8.2	1	3.5	8	4.6	10.25	33.1	tt
89	JAIKUMAR	42	M	66	12	0.8	8.9	1.2	3.8	6.7	4.6	17.85	21.8	tt
90	PREMKUMAR	35	M	98	14	0.7	11.3	1.1	3.5	7.2	2.9	22.97	65.5	tt
91	AROKYADAS	53	M	62	24	0.9	9.4	1.1	2.4	4.7	4.2	14.12	49.5	tt
92	JAMUNA	44	F	48	10	0.4	7.2	1.1	3.5	5.8	3.7	12.73	89.2	tt
93	DEVI	29	F	57	17	0.7	11.6	1.1	3.8	7	4.4	33.01	62.5	tt
94	SIVA.V	38	M	27	16	0.9	11.6	1.2	3.2	6.5	5.1	31.86	11.6	tt
95	NAGA	51	F	28	24	0.9	10.5	1.2	3.7	8.4	4.5	30.78	19.6	tt
96	VIJAYAN	39		46	18	0.8	9.5	1.2	4.4	7.1	4.5	23.71	55.1	tt
97	PRAKASH	34	M	38	23	1.2	9.3	1.1	3.4	6.8	3.8	39.35	36.3	tt
98	BALA.	28	M	53	20	0.6	9	1.1	4.8	7.6	4.2	27.21	51.1	tt
99	RAJKUMAR	54	M	90	23	1	9.7	1.1	3.1	7.1	4.5	22.14	63.3	tt
100	REKHA.	33	F	48	13	0.7	7.5	1.1	3.8	6.8	4.7	5.54	51.8	tt