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Molecular Evaluation and Linkage Analysis of Vitamin-D Deficiency in Female Population of Lahore

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Abstract

Vitamin D is a lipid soluble vitamin that is also known as CALCIFEROL, it is available as a nutritional supplement, naturally found in certain foods. There can be two conditions regarding vitamin D level in body, it can either get high or low. Hypovitaminosis D is more common among elderly women than men. So, for the recent studies prevalence of vitamin D deficiency was checked in women from random age group. Phylogenetic tree was constructed for the diversity linkages analysis of sequences used in the research and it revealed that the sequences showed great homology with the other species which is checked by boot strap method. Using docking tools the interactions between the vitamin D receptor protein and its ligand were also checked for the discovery of effective drug for the control of vitamin D deficiency on the genetic level. The research finding revealed that hypovitaminosis D is more prevalent in women because of the gestational complexities and poor nutritional count of the body. Previously the condition of vitamin D deficiency has been treated with vitamin D supplements and other medication but as per it's concluded that this is not the permanent solution of the problem.

Introduction

Vitamin D is a lipid soluble vitamin that is also known as CALCIFEROL. It is produced internally when the UV rays from the beam of sunlight directed toward the skin and speed up the vitamin D production. Foods, supplements and sun exposure are the sources that produces biologically inert vitamin D that needs to pass through hydroxylation for activation in the body. The hydroxylation eventually occurs first in liver that converts vitamin D into 25-hydroxyvitamin {25-(OH) D} which is called as "CALCIDIOL" and the second hydroxylation occurs in the kidney which converts vitamin D into 1, 25-dihydroxyvitamin D {1, 25(OH) 2D}, that can be called as "calcitriol" (Del *et al.,* 2011).

The vitamin D level can be indicated mainly by the serum concentration of 25(OH) D. It shows the amount of vitamin D obtained from an outsource and produced internally. [1] 25[OH] D has a long circulating half-life of 15 days in serum.1, 25(OH)2D in general is not a good indicator of

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vitamin D status as compared to 25(OH)D because it has a short life which can be measured in hours, and phosphate , calcium and parathyroid hormone tightly regulate the serum levels. Only severe vitamin D deficiency makes the 1, 25(OH) 2D level drop (Norman & Henry, 2012).

Location of Vitamin-D Gene

Faraco and his coworkers discovered the Apal dimorphism at the VDR locus thus the VDR gene was assigned to chromosome 12 by somatic cell hybrid studies (Faraco *et al.,* 1989).

Role of Vitamin-D

Vitamin D3 which is also known as cholecalciferol which is produced in the epidermis in reflection of ultraviolet radiation and the dietary vitamin D2 which is known as ergocalciferol, produced in plants, lack the biologic activity. Hormonal activity of vitamin D is regulated mainly due to the hydroxylated metabolite of vitamin D3, 1-alpha,25-dihydroxyvitamon D3(1,25(OH)2D3, OR Calcitriol). VDR (vitamin D receptor) is an intracellular hormone receptor that specifically binds 1,25(OH)2D3 and facilitate its effect (Baker *et al.,* 1998; Liberman *et al.,* 2001; Koren, 2006).

Vitamin plays an important role in bone turnover and in the regulation of calcium-phosphate homeostasis significantly. It also effects the skeletal health during growth process, its deficiency leads to rickets and also in adulthood (Antonucci *et al.,* 2018), in adulthood its deficiency results in osteomalacia and many degrees of osteoporo-malacia (Udey & Hogler, 2018). Vitamin plays an important role in the regulation of innate immunity, the very first data collected on this topic have been published on the treatment of tuberculosis and leprosy, diseases caused by mycobacteria (Airey F.S, 1946; Herrera G, 1949).

25(OH)D3 suppresses the adaptive immunity (Wei & Christakos, 2015, Chun *et al.,* 2014). 25(OH)D3 suppresses the immune responses regulated by T helper (Th) 1 cells in experimental models, thus prohibiting the synthesis of pro-inflammatory cytokines, such as interferon- γ, IL-6, IL-2 and TNF- α (Carvalho *et al.,* 2017; Xie *et al.,* 2017). 1,25(OH)2D3 supplementation plays an important role in control of autoimmune diseases as animal studies, such as experimental autoimmune encephalomyelitis(EAE) and collagen-induced arthritis(CIA). In both of these conditions 1,25(OH)2D3 prevents the onset and the reduces the progression(Lemire & Archer, 1991; Cantorna *et al.,* 1996; Cantorna *et al.,* 1998).

Vitamin D maintains the necessary serum calcium and promotes the calcium absorption in the gut and also maintains the phosphate level to enable normal bone mineralization and to prevent hypocalcaemic tetany (involuntary contraction of muscles, results in cramps and spasms). It is also important for the remodeling of bone by osteoblasts and osteoclasts and bone growth. Due to vitamin deficiency bones can become brittle, thin or misshapen. Vitamin D sufficiency can prevent osteomalacia in adults and rickets in children. Other roles of vitamin D in the body can be the reduction of inflammation and the regulation of cell growth, neuromuscular and immune function and metabolism of glucose (Del *et al.,* 2011; Norman & Henry, 2012; Jones G, 2014).

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Vitamin-D Deficiency and Comorbidity

Vitamin D low diets are more common among the people who have lactose intolerance or milk allergy and those who use up an ovo-vegetarian or vegan diet (Del *et al.,* 2011). Some observational studies elaborated the link between vitamin D deficiency and the risk of hypertension or cardiovascular events, more musculoskeletal pain or migraine, or cardiovascular events, higher incidence of cancers, and neuropsychiatric disorders such as dementia or depression, schizophrenia (Waydert, 2014).

Cancer

More than a three decades before, Colston and his coworkers elaborated that the span of melanoma cells increase after treatment with 1,25(OH)2D3 (Colsten *et al.,* 1981). Abe and his coworkers reported shortly after that HL60 leukemia cells differentiate towards the macrophage lineage upon incubation with 1,25(OH)2D3. Many studies have shown that 1,25(OH)2D3 and its analogs arrest cells in the G0/G1 phase of cell cycle by slowing down growth of cancer cells (Abe *et al.,* 1981).

Obesity

It has been determined that obesity is linked with vitamin D deficiency, providing a potential pathogenetic linkage with infertility (Holick *et al.,* 2011). Debate of the time is, whether the obesity contributes to the vitamin D deficiency or the deficiency contributes to the obesity. Even if the mechanism is not clear, it has been suggested that obesity can cause low level of vitamin D, due to its accumulation in adipose tissues leaving only small amount behind in the circulation (Rafiq, 2018). It is proposed that obesity may cause vitamin D deficiency due to improper intake or reduced exposure of obese people to UV radiation (Wortsman *et al.,* 2000). Moreover, the enzymes that catalyzes the hydroxylation of vitamin D to its active form produced in lower concentrations in obese when compared with non-obese patients (Wamberg *et al.,* 2012).

Covid-19

Stimulatingly, vitamin D deficiency can also be known in elderly and African Americans due to insufficiencies in vitamin D uptake, metabolism, and/or signaling (Holick *et al.,* 2011, Namgung *et al.,* 1994; Baeke *et al.,* 2010; Sarkar *et al.,* 2015). Some chronic disease conditions such as heart disease and diabetes are also linked to insufficient vitamin D levels, thus supporting a possible indirect link between vitamin D insufficiency and COVID-19 severity (Christakos *et al.,* 2013).

The link between COVID-19 severity and vitamin D deficiency is consistent with several historic, anecdotal and clinical studies linking insufficient vitamin D level to various infections, chronic and autoimmune diseases e.g. respiratory syncytial virus(RSV), tuberculosis(TB), HIV, HBV, HSV, Dengue Virus, malaria, leprosy, cancer, multiple sclerosis(MS) and inflammatory bowel disease(IBD) (Sarkar *et al.,* 2015; Studzinski *et al.,* 2015; Srinivasan *et al.,* 201; Cannell *et al.,* 2006) . Furthermore, an incidence of infection between seasonal influenza and vitamin D level is well known. (Aloia *et al.,* 2007; Goldstein *et al.,* 2010).

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Arthritis

The role of vitamin D in regulating immune function is subsidized by the discovery of vitamin D receptors (VDR) in peripheral mononuclear cells (Cantorna *et al.,* 2004; Delucs & Cantorna, 2001). Vitamin deficiency has been related to many autoimmune diseases, which includes insulin dependent diabetes mellitus, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (Jones *et al.,* 2008; Cantorna, 2000; Kamen *et al.,* 2006; Lips P, 2004).

Recently the role of vitamin D deficiency in the pathogenesis of RA , and the link between vitamin D deficiency and the activity of RA is discussed too(Song *et al.,* 2012). It is also stated that the risk of RA can be developed due to vitamin D deficiency (Merlino *et al.,* 2004). RA is an inflammatory disease characterized by flares and remissions, flares being characterized by pain. Vitamin D deficiency is also known to be associated with diffuse musculoskeletal pain (Hirani, V, 2012).

Literature Review

Vitamin D can be obtained from some dietary sources, but only these sources are not enough to provide sufficient amount of vitamin D (Holick MF, 2006). Sunlight provides 90% of the vitamin D. In adults, vitamin D deficiency can be defined as the serum 25-hydroxyvitamin D level less than 20ng per ml (50 nmol per L) and insufficiency can be defined as a serum 25- hydroxyitamin D level of 20 to 30 ng per ml (50 t0 75 nmol per L) (Holick, 2007).

Epidemiology

In industrialized cities vitamin D deficiency was the biggest cause of rickets in children in 19th century. This information led to the resolution of a major health issue linked with vitamin D deficiency and fortification of various foods. Although, it has been discovered that the vitamin D insufficiency and deficiency is directly linked with some pathologic conditions in all age group of people. There is an important of vitamin D in neuromuscular functioning, skeletal development and bone health maintenance

Prevalence rates of severe vitamin D deficiency, defined as 25(OH) D <30 nmol/L (or 12 ng/ml), of 5.9 %(US) (Schleicher *et al.,* 2016)**,** 7.4 %(Canada) (Sarafin *et al.,* 2015),13% (Europe) (Cashman *et al.,* 2016)have been reported. Estimated prevalence of 25(OH)D levels <50nmol/L (or 20ng/ml) have been reported as 24%(US), 37%(Canada), and 40%(Europe)(Cashman, 2016; Cashman, 2019; Schleicher *et al.,* 2016; Sarafin *et al.,* 2015).this percentage may vary with age, with lower levels in childhood and elderly(Cashman, 2019), and also ethnicity in different regions, for example , European Caucasians show lower rates of vitamin D deficiency in comparison with nonwhite individuals. Many countries across the world reports very high prevalence of low vitamin D status. 25(OH) D levels <30nmole/Lsarafin (or 12ng/ml) in >20% of the population are common in India, Tunisia, Pakistan and Afghanistan. For example, it has been estimated that 490 million individuals are vitamin D deficient in India (Cashman, 2019; Cashman, 2016). Patients on hemodialysis and with chronic renal failure, renal transplant recipients affected with liver disease or after liver transplantation may have a prevalence of

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vitamin D deficiency ranging from 85 to 99% (Courbebaisse *et al.,* 2014; Vos *et al.,* 2017; Zhou *et al.,* 2019).

Etiology

The major source of ergocalciferol D2 and cholecalciferol D3 are dietary intake (fatty fish livers, fortified food) and skin synthesis, and later these two are converted into 25-hydroxy-vitamin D2(25-OH-D2) and 25-hydroxy-vitamin D3(25-OH-D3) respectively in the liver by the enzyme hepatic enzyme 25-hydroxylase.

Decreased dietary intake and/or absorption

A few malabsorption syndromes which can lead to vitamin D deficiency includes short bowel syndrome, gastric bypass, inflammatory bowel disease, chronic pancreatic insufficiency, celiac diseases and cystic fibrosis. In elderly population lower vitamin D intake is more common (Czernichow et al., 1667).

Decreased sun exposure

About 50 to 90% of vitamin D can be obtained from sunlight and the rest comes from diet. Daily sunlight exposure of 40% skin of almost twenty minutes is required to prevent vitamin D deficiency (Naeem, 2010).Cutaneous synthesis of vitamin diminishes with aging. Cutaneous vitamin D synthesis less in dark skinned people. People who are institutionalized or have prolonged hospitalization can be seen with less sun exposure which can also leads to vitamin D deficiency (Thomas et al., 1998).Effective sun exposure is decreased in individuals who use sunscreens persistently.

Decreased endogenous synthesis

Individuals who have chronic liver disease such as cirrhosis can have defective 25-hydroxylation leading to deficiency of active vitamin D. Defect in 1-alpha 25-hydroxylation can be seen in hyperparathyroidism, renal failure and 1-alpha hydroxylase deficiency.

Increased hepatic catabolism

The medications which regulate hepatic p450 enzymes to activate degradation of vitamin D includes clotrimazole, spironolactone, nifedipine, dexamethasone, carbamazepine, phenobarbital and rifampin (Grober & kisters, 2012).

End organ resistance

End organ resistance to vitamin D can be seen in hereditary vitamin D resistant rickets.

Pathophysiology

Vitamin D plays an important role in calcium homeostasis and bone metabolism. With chronic and /or severe vitamin D deficiency, a reduction in intestinal calcium and phosphorus absorption leads to hypocalcaemia leading to secondary hyperparathyroidism. Later on secondary

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hyperparathyroidism then leads to phosphaturia and regulated bone demineralization. This can also results in osteomalacia and osteoporosis in adult and osteomalacia and rickets in children.

Pathogenesis

Normal bone metabolism is changed without the presence of activated vitamin D, so that only 10 % of calcium and 60 % of phosphorus is absorbed (Holick, 2007).In a result, the skeleton becomes the body's primary source of calcium (Holick, 2003),with osteoclasts dissolving bone to raise serum calcium (Holick & Garabedian, 2006).These actions can lead to osteomalacia, and they precipitate and exacerbate osteopenia and osteoporosis.

Symptoms

Most vitamin D deficiency patient do not show symptoms. Moreover, sometimes mild chronic vitamin D deficiency can cause chronic hypocalcaemia and hyperparathyroidism which can increase the risk of osteoporosis, falls and fractures specifically in elderly population. People who are suffering from severe vitamin D deficiency from a long time can also experience symptoms related to secondary hyperparathyroidism including bone pain, myalgias, arthralgias, bone pain, muscle twitching (fasciculation), fatigue and weakness. Vitamin D deficiency can result into fragility fractures leading to osteoporosis.

Manifestation of vitamin deficiency

1. Muscle aches
2. symmetric low back pain in women
3. increased risk of falls and impaired physical function
4. proximal muscle weakness
5. bone discomfort or pain (often throbbing) in low back, pelvis, lower extremities (Hicks *et al.,* 2008, Holick 2007)

Risk Factors

Use of medication can cause vitamin D deficiency e.g. anticonvulsants or glucocorticoids, that can speed up catabolism and efficiently destroy vitamin D (Holick, 2007).Nevertheless, approximately one third of person with known deficiency have no identifiable risk factors (Schneider 2006).For example, in a study of 142 healthy persons, most of whom consumed milk and supplements, participants18 to 19 years of age with no risk factors for deficiency were identified to have the lowest levels of vitamin D.

Risk factors for vitamin D deficiency **(Holick 2003, Schneider 2006)**

Use of medication that can alter vitamin D metabolism(e.g. anticonvulsants, glucocorticoids
Sedentary lifestyle
Obesity(body mass index greater than 30kg per m2)
Age older than 65yrs
Breastfed exclusively without vitamin supplementation
Dark skin
Insufficient sun exposure

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Vitamin-D Deficiency in Pregnancy and Fetal Programming

During pregnancy and lactation, changes occurs in calcium and vitamin D metabolisms to meet the demand for fetal bone mineralization. The fetus accumulates 2-3mg/day of calcium in the skeleton during the first trimester, but it doubles in the last trimester (Schneider, 2006).The pregnant woman's body adapts the fetal needs and increases calcium absorption in early pregnancy. This calcium transfer is balanced by more intestinal absorption and less urinary excretion of calcium.

1,25(OH)2 D level increases in blood plasma in early pregnancy, which reaches to its peak in the last trimester and during lactation it returns to normal. Considering that PTH levels do not change during pregnancy, the stimulus for increased production of 1, 25(OH) 2D is unclear (Mulligan *et al.,* 2010).

Present studies stress upon the importance of non-classical roles of ViD during pregnancy and in the placenta and correlate VDD in pregnancy with insulin resistance, preeclampsia, gestational diabetes, bacterial vaginosis and increased frequency of cesarean delivery (Kaushal & Megan, 2013).

When compared to women with normal level of ViD, a novel study showed that cesarean delivery is four times more common in women with VDD (<37.5 nmole/L) (Merewood *et al.,* 2009).One of the major risk factor of VDD in childhood is maternal VDD, as in the first 6-8 weeks of life newborns depends on the ViD transferred across the placenta while in the womb. This association is linear and the 25(OH) D levels of the newborn direct to 60-89% of maternal level (Dawodu & Wagner, 2012).

Staging

Mild deficiency	25-hydroxyvitamin D < 20ng/mL
Moderate deficiency	25-hydroxyvitamin D < 10ng/mL
Severe deficiency	25-hydroxyvitamin D < 5ng/mL

The severity of vitamin D deficiency is divided into three stages (Holick & Garabedian, 2006)

Diagnostic Evaluation

25-hydroxyvitamin D is the main indicator of vitamin D status because it the major calculating form of vitamin D. and it also reflects the cutaneous and dietary contributions, it is also considered to be a precursor for 1,25-dihydroxyvitamin D, the most active vitamin D metabolite (Holick, 2006).1,25(OH)2 D metabolite should not be used for calculating vitamin D levels because these levels can be increased by secondary hyperparathyroidism (Holick, 2007). Physician should recommend those patients for vitamin D testing who show unexplained symptoms.

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Quantification of Vitamin-D Deficiency

To measure 25(OH)D levels many methods have been developed which includes high performance liquid chromatography(HPLC), chemiluminescence, and radioimmunoassay (RIA).

LC-MS/MS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered the "Gold standard" for detection and quantification of many analytes, it is a highly sensitive technique. Some methods of LC-MS/MS are used for the quantification of 25(OH) D2 and 25(OH) D3; even so, all require derivatization and/or expensive transitive internal standards (Eisman *et al.,* 1977). For example, the 25(OH) D LCMS/MS method described by Higashi and his coworkers had no sample pretreatment requirements but required the use of derivatization methods with a Cooksontype reagent. Tsugawa and his coworkers introduced a liquid chromatography-atmospheric pressure chemical. The later methods were lacking on complex synthesis of the internal standard and relatively long chromatographic time (11 minutes) (Tsugawa *et al.,* 2005). Vogeser and his coworkersdid some modifications in the extraction procedure by using solid-phase extraction to obtain further purification before quantification of 25(OH) D2 and 25(OH) D3 (Vogeser *et al.,* 2004). In present, Maunsell and his coworkers used isotopes-dilution LC-MS/MS for detection of 25(OH)D2 and 25(OH)D3 using a deuterated vitamin D3 internal standard with longer analysis time(8 minutes)ionization mass spectrometry method using deutesrated 25(OH)D3 a the internal standard (Maunsell *et al.,* 2005).

Differential Diagnosis

- Celiac sprue
- Use of antiepileptic medication
- Lack of dietary intak2
- Insufficient sunlight exposure
- End-stage liver disease

Treatment and Prevention

The institute of Medicine has recommended adequate intake (AI) based on levels required to maintain optimal bone health in all members of a healthy population to prevent vitamin D deficiency in person with improper sun exposure. The present daily AI is 200 IU for infants, children ,adults younger than 51 years;400 IU for adults 51 to 70 years of age; and 600 IU for adults older than 70 years (Cranney *et al.,* 2007; Holick MF 2006). Anyhow, current AI recommendations for children and adults according to current research may be too low to maintain optimal levels (above 30ng/mL) for calcium absorption and parathyroid hormone suppression (Norman *et al.,* 2006; Bischoff *et al.,* 2007).The American academy of pediatrics based on these concerns recently recommended doubling the minimum daily intake for children and adolescents to 400 IU.

Research Objectives

Objectives includes;

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- To determine the prevalence rate and diversity linkage of vitamin deficiency in female population
- To purpose the therapeutic drug/genetic therapy through molecular docking.
- The analysis of related comorbidities and their implications on women health.
- Identification of the target gene and its protein expressions.

Material and Methodology

Sample Collection

We used blood samples of random females with no age and disease specifications in our research.

Fifty samples were collected from different laboratories of Pakistan with people's consent. Blood samples were drawn from subjects by an experienced technician into a 50ml tube through medical syringe containing 200ul ethylene-di-amine tetra acetic acid as an anti-coagulant. A code number was assigned to each blood sample and to prevent it from clotting, tubes were spin many times for absolute mixing of anti-coagulant.

Molecular analysis of DNA

Isolation and analysis of DNA was done by molecular identification of DNA by the DNA Extraction.

DNA Extraction

Genomic DNA extraction was carried out on stored blood sample by DNA extraction kit and standard organic DNA extraction method.

Organic Extraction

In molecular biology the most basic of all procedures is the purification of DNA. In organic extraction, removal of protein which is the main step can be carried out by extraction the aqueous solutions of nucleic acids using phenol or chloroform.

Procedure of DNA Extraction (Manual method)

- Stored blood samples were thawed by putting the EDTA vials at room temperature in water bath.
- For the lysis of RBCs in 500ul blood was taken, 500ul of lysis buffer (TE) was added in it and then it was mixed by inverting several times.
- Tubes were then centrifuged at 4℃ at 14,500 rpm for 10 minutes.
- A pallet was formed at the bottom after centrifugation, discarded the supernatant containing the flow through solution and by tapping the pallet gently break the pallet.
- TE buffer was added again followed the centrifugation step again until pallet become white.
- 500ul of TE buffer and 10ul of proteinase K were added. Contents were mixed gently for 10 minutes and then tubes were centrifuged at 14,500 rpm for 10 minutes.

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- The top aqueous phase containing the DNA was carefully removed by using the pipette of 1000ul and transferred the aqueous phase into a new (tube) eppendorf.
- Tubes were then incubated overnight at 45-50℃ in water bath for complete digestion of cellular protein.
- Next day, 500ul of PCT (phenol, chloroform, isoamylalcohol, 25:24:1) was added and tubes were centrifuged after that at 4000 rpm at 4℃ for 20 minutes. This will split the tube components into two layers, lower layers consisting of salts and proteins and upper layer consisting of DNA in soluble form.
- The upper aqueous layer was carefully transferred into new labelled eppendorfs.
- Chilled ethanol of equal volume (500ul) was added to the eppendorfs. Then the tubes were smoothly inverted until the DNA threads were become visual.
- Tubes were incubated at room temperature for 10 minutes.
- Tubes were centrifuged at 4000 rpm, at 0℃ for 5 minutes. Supernatant was discarded carefully after centrifugation.
- With 500ul of 70% ethanol, DNA pallet was washed for 2 hours in the incubator shaking stand and then tubes were centrifuges at 4000 rpm, at 0℃ for 5 minutes.
- Ethanol was discarded after carefully saving the DNA pallet.
- Then previous step was repeated, DNA pallet was washed again for 2 times.
- Air dried the DNA pallet in an incubator at 37℃ till ethanol smell was finished.
- According to the amount of DNA pallet low TE buffer 50ul was added in the Eppendorf. Then the DNA was gently dissolved in low TE buffer.
- Eppendorfs were labelled on the sides and caps properly.
- Extracted DNA samples were stored at -20℃ for the further us

Procedure for extraction of DNA by using Kit (Automated method)

- Thermo scientific Gene JET Genomic DNA purification Kit was used for automated DNA extraction.
- Stored blood samples was thawed after this 200ul of blood was taken in a tube and 400ul of lysis solution, 20ul of proteinase K was added in the tube. To obtain a uniform suspension contents of the tube were mixed properly by vortexing.
- Samples were incubated at 56℃ in a shaking incubator at low speed for 10 minutes to lyse the cells completely.
- Then 200 ul of 96% ethanol was added to the tubes and mixed by vortexing.
- The prepared lysate was transferred to the GeneJET Genomics DNA Purification column which was inserted in a collection tube. Column was centrifuged at 600 rpm for 1 minute and then the collection tube wads discarded containing the flow through solution.
- Purification column was inserted into a new collection tube, provided by the Kit.
- 500ul of washing buffer I was added to the column and then centrifuged at 8000 rpm for 1 minute. Flow through was discarded and purification column was inserted back to the collection tube.
- 500 ul of washing buffer Π was added to the purification column and centrifuged at maximum speed (15000 rpm) for 3 minutes. Collection tubes was then discarded containing

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the flow through solution and then transferred the purification column to a autoclaved 1.5ml eppendorf tube and then labelled the tube.

- To elute genomic DNA, 200 ul of elution buffer was added to the center of the purification column membrane, then incubated at room temperature for 2 minutes.
- Centrifuged at 8000 rpm for 1 minute. Purification column was discarded carefully.
- Purified DNA was stored at -20℃ for future use.

Gel Electrophoresis

Genomic DNA analysis of extracted DNA was performed by gel electrophoresis. Confirmation of DNA was done by mixing 1 g of agarose gel and DNA was loaded into the wells of gel.

Gel was run at 90 V and 250 AMP to visualize bands in gel dock.

PCR (Polymerase Chain Reaction) Profile

Polymerase chain reaction was done and VDF1, VDR1, VDF2, VDR2 were optimized and DNA sample were amplified

Designed Primers used for amplification

Primers	(Forward/Reverse)	Sequence	Temp. ℃	product
VDF1	(F)	AGGACAGCTTTCAGGTCTATGG	58	298
VDR1	(R)	GATATTCCCGTTTCCAACGA	53	
VDF2	(F)	TGGAAGTGAACATTAGGACAGC	57	315
VDR2	(R)	GATATTCCCGTTTCCAACGA	53	

Sequencing of DNA samples

To examine DNA samples, sample VDF1-1, VDF2-2, VDF1-3, VDF2-4 were sent to the special service for sequencing to the first base laboratories, Selangor, Malaysia. PCR tubes were labelled properly by sides and caps or the tubes and then by using parafilm tape, tubes were covered to avoid any leakage or evaporation of the product. Tubes were then placed in labelled polythene zip lock bag and sent for sequencing.

Primer Designing

Primer used in the research were made with PRIMER 3 software.

Insilico and Bioinformatics work

Phylogenetic analysis

VDF1 , VDF2, VDR1, VDF2 sequence were aligned using BLAST and MEGA X was used , by selecting neighbor joining tree sequences were aligned and phylogenetic tree was formed the check the diversity linkage.

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Molecular Docking

PatchDock online software was used for molecular docking to analyze protein and ligand Interactions.

Tools used for PatchDock

1. Protein Data Bank (PDB)

Protein data bank was used to find the 3D structure of protein and ligand and then downloaded in PDB format.

2. PyMol 2.5

PyMol used as a visualizer for protein 3D structure.

Protein-Ligand Interaction Profiler (PLIP)

Used for the characterization of interaction between the protein and ligand complexes.

3. PartchDock

It is used for the online docking of the protein receptor and ligand molecule

Collection of samples

Blood samples of random females with no disease and age group specifications from different territory of Pakistan for research reasons.

DNA Extraction Results

DNA extraction was done with samples 1,2,3,4,5,6,7 by manual DNA extraction and extraction kit as well. Presence of DNA in the samples were confirmed.

Extracted DNA result of sample 1,2,3,4,5,6,7

Polymerase chain reaction (PCR) Results

PCR results were obtained. By using VDF1, VDR1, VDF2 and VDF2 primers genes were amplified.

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PCR amplified results of sample no 1,2,3,4

Sequencing Blast Results

VDF1, VDR1, VDF2, VDR2 sequences of different blood samples were used in BLAST for the identification and to check similarities between our sequences and other sequences and to find the ID of gene.

Molecular identification of VDF1, VDR1, VDF2, VDR2 sequences and submission to NCBI GenBank

Sr. no.	Sample Number	Location	Source	Genus	%age Identity	Query Coverage	Accession No.
1	1	Lahore	Human blood	*Homo sapiens*	96.27%	93%	AC270172.1
2	2	Lahore	Human Blood	*Home sapiens*	99.81%	97%	CP068256.2
3	3	Lahore	Human Blood	*Home sapiens*	92.40%	93%	CP068256.2
4	4	Lahore	Human Blood	*Homo sapiens*	91.61%	99%	CP068256.2

Bioinformatics Tools

1. Phylogenetic Tree

After analysis, information was given that linked to evidence and the relation evolutionary history of species. Three categories were involved; maximum linked tree, neighbor joining tree and minimum evolution tree. 1,2,3,4 sequences showed the similarities, showed in phylogenetic tree. By using MEGA X bootstrap method was used and about 500 replication of bootstrap were applied.

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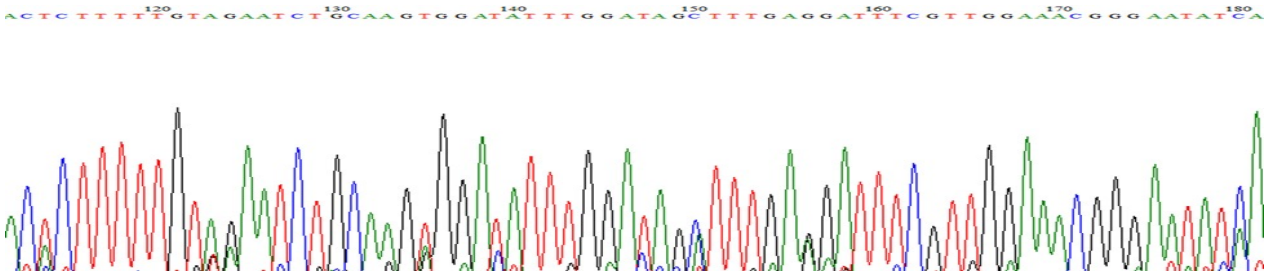
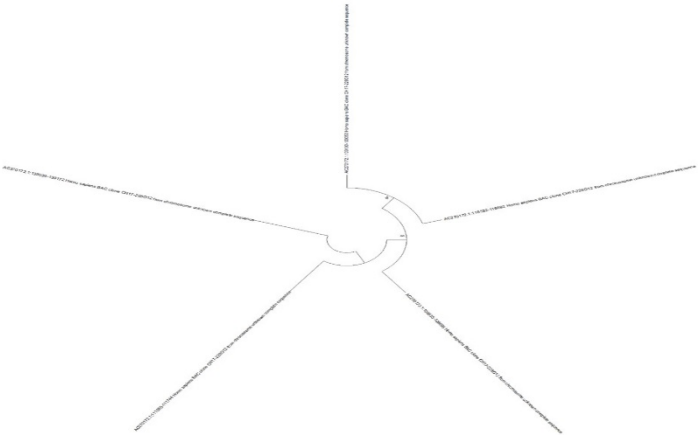
Chromatogram and phylogenetic tree of 1-VDF1 sequence

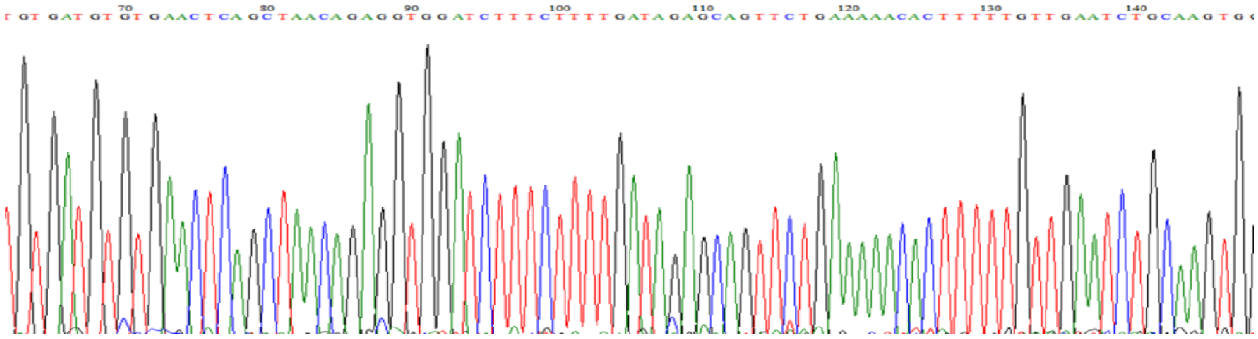


(a) Chromatogram of 1-VDF1 after sequencing

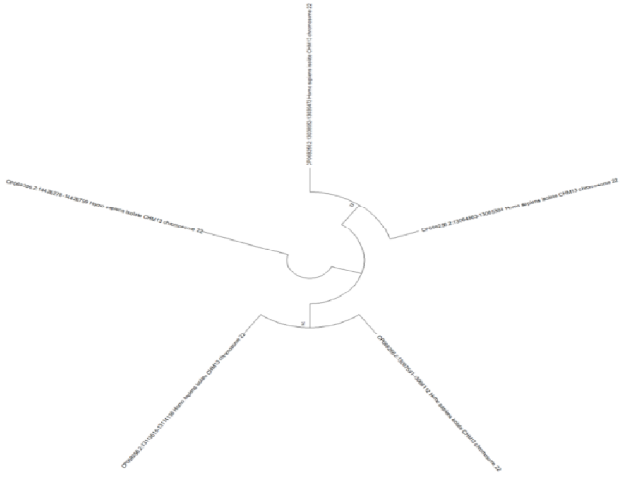


(b) Neighbor joining phylogenetic tree of that shows the evidence and evolutionary distance between same species, branches with number shows the bootstrap value.

Chromatogram and phylogenetic tree of 2-VDF2 sequence



(a) Chromatogram of 2-VDF2 after sequencing



(b)Neighbor joining phylogenetic tree of that shows the evidence and evolutionary distance between same species, branches with number shows the bootstrap value.

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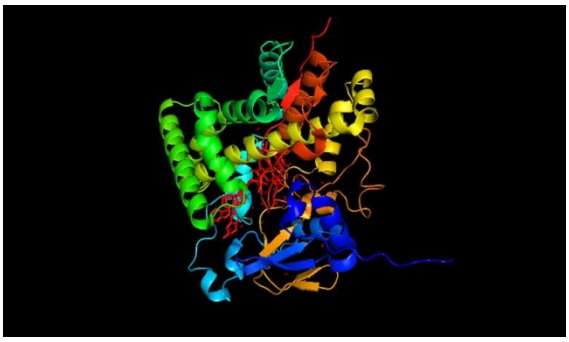
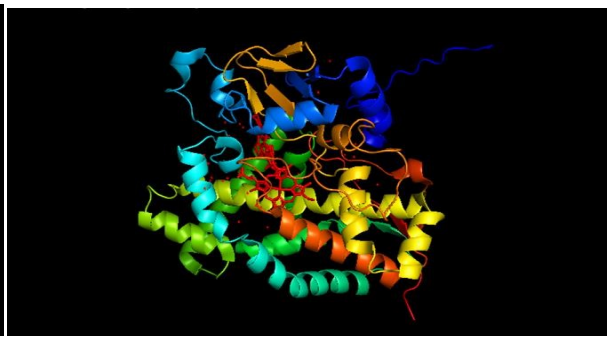
Molecular Docking

Molecular docking was done by PatchDock to speculate molecules when bound top each other. Docking used to explore protein and ligand interactions. 3D structure were predicted by PDB and their results were anticipated by using PyMol software.



Protein receptor 3D structure



3D structure of ligand HEM

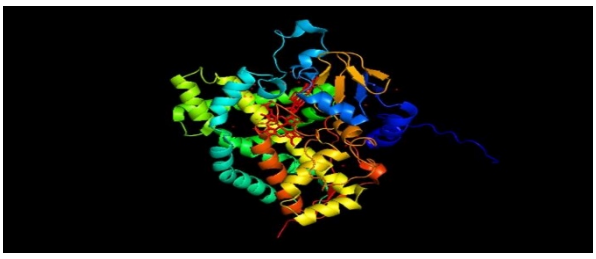
Molecular Docking examination

By using PatchDock, vitamin D receptor protein and ligand molecule structure was uploaded. Email address was added and clustering RMSD value was kept 4.0 by default then form was submitted. Results showed highest atomic contact energy value, which was 271.96.

Solution No	Score	Area	ACE	Transformation	PDB file of the complex
1	16842	2000.10	271.96	-2.74 0.83 -0.88 -9.36 49.96 56.73	result.1.pdb
2	16040	1899.40	204.40	1.99 -0.11 -2.68 77.84 1.40 16.22	result.2.pdb
3	15966	2433.90	-168.43	-2.66 -1.10 1.12 22.38 55.88 80.79	result.3.pdb
4	15902	2236.30	-83.66	0.87 -0.81 2.92 70.56 13.63 36.40	result.4.pdb
5	15884	2100.40	233.38	1.23 1.16 2.22 -37.12 26.06 -41.80	result.5.pdb
6	15372	2135.20	154.93	-0.65 0.47 2.43 69.74 43.78 -10.79	result.6.pdb
7	15318	2316.90	317.03	1.47 1.33 2.05 20.50 48.05 33.62	result.7.pdb
8	15190	1958.30	370.87	-3.11 -1.42 -0.92 14.79 53.85 34.60	result.8.pdb
9	15118	2261.50	-61.65	1.14 -0.20 -2.42 71.61 30.20 -9.76	result.9.pdb
10	15090	2201.90	404.53	-0.52 -1.22 2.81 22.32 28.42 -8.05	result.10.pdb
11	14902	2171.70	442.98	1.21 0.16 -0.72 -26.19 30.42 -14.04	result.11.pdb
12	14892	2140.70	431.04	-1.14 -0.76 0.08 42.36 5.22 9.20	result.12.pdb
13	14694	2579.20	-374.02	-2.38 -0.76 1.09 38.99 42.67 81.10	result.13.pdb
14	14684	2144.10	177.89	2.06 0.16 1.71 19.72 -0.67 -4.15	result.14.pdb
15	14680	1832.10	214.80	-0.64 0.60 -1.81 -51.26 22.88 20.10	result.15.pdb
16	14536	1969.90	138.22	-1.48 -0.14 -0.31 37.93 -1.13 29.08	result.16.pdb
17	14534	1991.20	-216.47	-3.13 0.41 -0.33 -8.64 26.74 59.41	result.17.pdb
18	14500	1974.60	421.01	1.75 -0.53 -1.36 36.87 41.69 -31.57	result.18.pdb
19	14426	2020.90	53.55	3.11 1.03 0.10 -22.45 15.59 38.41	result.19.pdb
20	14422	2143.40	180.83	2.51 0.17 2.79 6.32 -23.83 48.39	result.20.pdb

show next 20 >>

Highest energy score by molecular docking



Protein-Ligand interaction 3D structure

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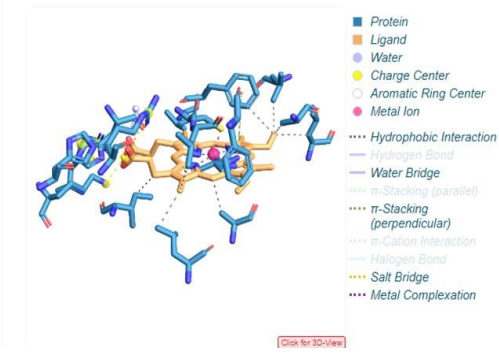
To analyze protein-ligand interaction, PLIP (protein-ligand profiler) tool was used by uploading the protein-ligand interaction structure in a PDB file. Results showed that protein structure has 2 binding sites; first one is small molecule HEM and the second is VD3.



Small molecule HEM and its interacting points

Hydrophobic Interactions

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	88A	ILE	3.54	3140	696
2	106A	PHE	3.72	3134	846
3	232A	LEU	3.57	3139	1812
4	236A	ALA	3.48	3110	1841
5	244A	LEU	3.85	3127	1898
6	277A	LEU	3.80	3127	2150
7	283A	VAL	3.55	3125	2200
8	312A	LEU	3.48	3115	2411
9	340A	PHE	3.78	3127	2627
10	348A	LEU	3.73	3139	2690
11	348A	LEU	3.95	3136	2687
12	353A	ALA	3.89	3127	2721

Water Bridges

Index	Residue	AA	Dist. A-W	Dist. D-W	Donor Angle	Water Angle	Protein donor?	Donor Atom	Acceptor Atom	Water Atom
1	345A	HIS	3.46	2.84	144.60	104.31	×	3120 [O.co2]	2659 [O2]	3225

Hydrogen bonding and water bridges in small molecule HEM

π-Stacking

Index	Residue	AA	Distance	Angle	Offset	Stacking Type	Ligand Atoms
1	340A	PHE	4.79	74.89	0.95	T	3121, 3122, 3123, 3124, 3146

Salt Bridges

Index	Residue	AA	Distance	Protein positive?	Ligand Group	Ligand Atoms
1	65A	LYS	3.66	✓	Carboxylate	3119, 3120
2	95A	HIS	4.10	✓	Carboxylate	3143, 3144
3	99A	ARG	4.74	✓	Carboxylate	3143, 3144
4	289A	ARG	3.38	✓	Carboxylate	3119, 3120
5	345A	HIS	4.07	✓	Carboxylate	3143, 3144

Metal Complexes

Index	Residue	AA	Metal	Target	Distance	Location
Complex 1: Fe, trigonal.bipyramidal (5)						
1	347A	CYS	3149	2682	2.38	protein.sidechain
2	501A	HEM	3149	3145	1.96	ligand
3	501A	HEM	3149	3146	2.11	ligand
4	501A	HEM	3149	3147	1.94	ligand
5	501A	HEM	3149	3148	2.12	ligand

π-stacking, salt bridges and metal complexes present in HEM

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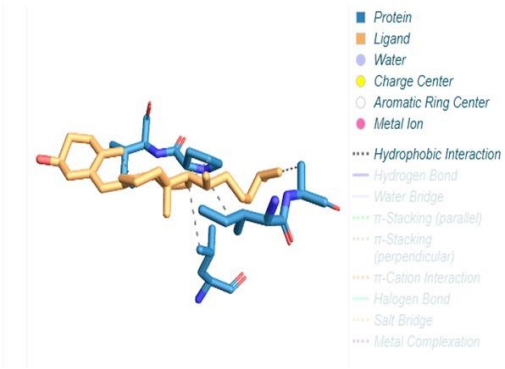

Hydrophobic Interactions

Index	Residue	AA	Distance	Ligand Atom	Protein Atom
1	235A	ILE	3.67	3192	1835
2	236A	ALA	3.54	3202	1841
3	287A	PRO	3.81	3182	2226
4	288A	ILE	3.73	3188	2235
5	387A	LEU	3.85	3182	2988

Small molecule VD3 and its interaction sites

Discussion

Hypovitaminosis D is more common among elderly women than men. So, for the recent studies prevalence of vitamin D deficiency was checked in women from random age group. For analysis of vitamin D gene expression two set of primers were used.

Similarity between the sequences were checked with BLAST and got the percentage identities of samples 1,2,3,4 as 96%, 99%, 92% and 91% respectively.

Phylogenetic tree was constructed for the diversity linkages analysis of sequences used in the research and it revealed that the sequences showed great homology with the other species which is checked by boot strap method. Phylogenetic analysis predicted accurate results using 500 bootstrap repeats and it proved same results when matched with blast sequence. Percentage identity of 1. 2, 3, 4 was 96%, 99%, 92% and 91% respectively.

Docking by Patch dock tool revealed protein and ligand interaction of vitamin D3 25-hydroxylase protein and HEM inhibitor as a ligand from highest atomic energy value 271.96. Protein ligand profiler identified two binding sites of protein named, a small mole which is HEM (protoporphyrin IX) and other is VD3 (cholecalciferol). Results also revealed their interacting points as hydrophobic interactions, hydrogen bonding, π stacking forces and metal complexes with residue and related amino acids. By using these binding sites and interactions between and inhibitor, an efficacious drug against the auto immune hypovitaminosis D disease can be constructed.

Conclusion

The research finding revealed that vitaminosis D is more prevalent in women because of the gestational complexities and poor nutritional count of the body. Previously the condition of vitamin D deficiency has been treated with vitamin D supplements and other medication but as per it's concluded that this is not the permanent solution of the problem. This research revealed that there must be other ways to prevent the loss. The molecular docking demonstrated interaction between inhibitor and protein receptor which shows some binding sites that can be modified genetically according to the need, it can help to eradicate the root cause of

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hypovitaminosis D that runs in the family. In future, more binding sites can be revealed to modify genes or regulatory protein which are involved in the production of vitamin D.

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