# An Etiological Study of Vitamin D Deficiency in Pakistani Population


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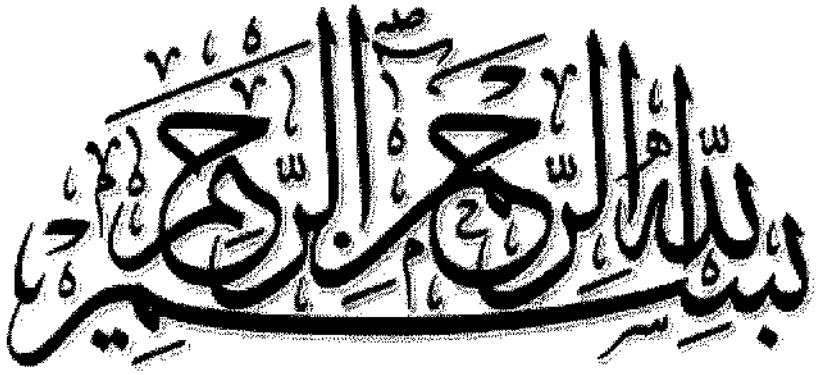
**(2015)**

- Pharmacology
- vitamins.



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Dated: 21-08-15

# FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms. Hina-Ur-Razaq Qureshi and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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**A thesis submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as a partial fulfilment of requirement for the award of the Degree of MS Biotechnology**

*DEDICATED*

*TO*

*MY*

*BELOVED*

*PARENTS*

*AND*

*MY FAMILY*

# DECLARATION

I hereby declare that the work present in the following thesis is my own effort, Except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 21-08-15

Hina-Ur-Razaq-Qureshi

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# ACKNOWLEDGMENT

All praises and thanks to ALLAH Almighty-Who is the ultimate source of knowledge to mankind Who bestowed man intellectual power and understanding to discover his "Self" and to know his "Creator" by discovering His signs extended throughout the universe-Who made me reach at present pedestal of knowledge with quality of doing something adventurous, novel, thrilling, sensational, and path bearing.

I take this opportunity to thank all those who have helped me in this research study. First of all, I would like to express my deepest appreciation and most heartfelt gratitude to my supervisor Dr. Naveeda Riaz (Chairperson, Department of Bioinformatics And Biotechnology, IIUI, Islamabad) for her keen interest and persistent encouragement. I always found her loving all her students like her own children.

I wish to express my heartiest obligation and sincere regards to my co-supervisor Dr. Uzma Shaukat (SSO IBGE, KRL, Islamabad) for giving me opportunity to work with her and also giving me learned guidance and skilled advice.

I am deeply grateful and indebted to Dr Anila (FCPS, KRL Hospital Islamabad), who cooperated with me in providing my concern patients for blood samples. I feel great honour and pleasure in expressing special thanks to Dr Umer Farooq (Head of Pathology Lab, KRL) who allowed me to stay in Pathology Lab for blood samples collection. I would particularly like to express my profound thanks to whole Pathology Lab Staff Mr Waseem, Mr Mobeen, Mr Rashid Aziz, Mr Shahid Iqbal their kind attitude and help in collecting blood samples is unforgettable.

Words are inadequate to express my deep sense of indebtedness and gratitude to my friends at KRL Lab 307 especially Bilal Ahamd, Huma Arshad, Fazal-ur-Rehman for their friendship, suggestions and generous help.

At last but not the least I want to give gratitude to all my friends Nazia Ali, Sarwat Zareen, Sumiya Abbasi, Nagina Atlas for their valuable friendship and wonderful support. I am deeply grateful particularly to Ayesha Rahim and Bilal Ahmad for their kind cooperation, constructive criticism, and valuable suggestions during the progress of my research and in writing up this manuscript.

Finally, I can't find words to express my thanks to my parents, my brothers and my sister in laws for their prayers, divine love and moral support to complete this study.

# LIST OF ABBREVIATIONS

| Acronym | Abbreviation |
|---|---|
| µl | Microliter |
| ng | Nanogram |
| BMI | Body Mass Index |
| Vit. D | Vitamin D |
| UVB | Ultraviolet B radiation |
| IU | International Units |
| 25(OH)D | 25-hydroxyvitamin D |
| $1,25(OH)_2D$ | 1,25-dihydroxyvitamin D |
| CYP27B1 | Cytochrome P450, Family 27, Subfamily B and Polypeptide 1 |
| RXR | Retinoid X receptor |
| $Ca^{+2}$ | Calcium ion |
| CYP24A1 | Cytochrome P450, Family 24, Subfamily A and Polypeptide 1 |
| CYP2R1 | Cytochrome P450, Family 2, Subfamily R and Polypeptide 1 |
| VDR | Vitamin D receptor gene |
| BMD | Bone mineral density |
| SPSS | Software Package Used For Statistical Analysis |
| SNP | Single Nucleotide Polymorphism |
| SDS | Sodium Dodecyl Sulphate |
| TBE | Tris-Borate-EDTA |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| EtBR | Ethidium Bromide |
| APS | Ammonium per Sulphate |
| OD | Optical Density |
| TBE | Tris Boric EDTA |
| TEMED | Tetramethylethylenediamine |

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# ABSTRACT

Vitamin D belongs to seco-steriods group and is involved in calcium and phosphorous absorption in intestine. Vitamin D can be acquired in human body by three main sources i.e. sunlight, diet and supplements, though the sunlight is chief source. Solar UVB radiation is absorbed in skin where precursor cholesterol molecule is transformed into previtamin D. Vitamin D deficiency can lead towards various diseases like osteoporosis, osteoarthritis, multiple secrosis, type I diabetes, depression, dementia, cancer, psoriasis, cardio-vascular diseases and muscle weaknesses. Dementia a neurological disorder is found to be associated with vitamin D deficiency. NOTCH3 gene of dementia is not involved directly in vitamin D metabolic pathway so on the basis of dementia link with vitamin D deficiency we further correlated the relation of NOTCH3 gene in our selected vitamin deficient family. Genotyping was done for linkage analysis of NOTCH3 gene. Genotyping results of the present study revealed that affected individual exhibited allele 2 which showed dominance inheritance pattern but selected marker was not linked with selected vitamin D deficient family as when dominant allele 2 was present in normal individuals of the family, it was not causing vitamin D deficiency in them. CYP27B1 is the gene involved in production of the bioactive molecule of vitamin D i.e. $1,25(OH)_2$ D so SNP rs 10877012 of CYP27B1 gene was selected to sequence the SNP rs 10877012 in cases samples and these samples were categorised according to the deficiency levels (deficient/insufficient/normal) and the genetic differences were observed in these categories with the help of sequencing. The sequencing results are under process. Furthermore, clinical assessment of the all patients were analysed by using statistical tool named SPSS 20. Statistical analysis of current study showed that females (82%) ration was high than the males (18%). Similarly, the ratio of females having deficiency of vitamin D is more dreadful than males. The calculated mean age of onset for vitamin D deficiency was 44 years. Sheikh and Pathan caste were more affected than the rest of the documented caste. The

high percentage of inadequate sunlight exposure was observed i.e. 46% and only 1% patients had good sunlight exposure which highlighting the fact that people needs sufficient sunlight exposure in order to maintain adequate vitamin D level in blood. Therapy response of vitamin D supplements was positive in current data so people should provide awareness about the benefits of these supplements so that vitamin D level can easily retain. Gender comparison with different age groups demonstrated that every age group was showing the high ratio of females. Arthiritis, osteoporosis, diabetes mellitus, backache, backbone issues, joint pains and depression were found to be the most occurring diseases/problems in present study. Keeping these above facts in mind it can be concluded that, we need consistent sufficient exposure to sunlight with large exposed area to get the desired amount of vitamin D (especially females) and with the help of genetic screening of families we can analyse the chance of occurrence of vitamin D deficiency in a generation and the genetic counselling helps to cope with this deficiency.

# INTRODUCTION

# 1 Introduction

## 1.1 Vitamin D and its Structure

Vitamin D is fat soluble molecule and reside under the seco-steriods class and involve in phosphate and calcium absorption in intestine. In 1922 vitamin D was firstly recognised and generally its existence is in two forms named as Vitamin D2 and Vitamin D3 (Figure 1.1) (Holick, 1994; Khan *et al.*, 2013). Vitamin D2 also known as ergocalciferol is the ending product of invertebrates like fungi and plants whereas Vitamin D3 or cholecalciferol is resulting from animal sources (Holick *et al.*, 2011). These two forms collectively named as calciferol. Vit. D2 and vit. D3 both are present in nutritive supplements and fortified foods. Although the major metabolic steps involved in synthesis of these two vitamin D forms are similar but still these two forms are biologically inert and further modifications are require for the production of 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) which is active product of vitamin D (DeLuca, 1988; Reichel *et al.*, 1989). The structural difference of vitamin D2 and vitamin D3 is in their side chains i.e. the presence of methyl group at carbon 24 of vit. D2 and carbon 22 and carbon 23 is connected via double bond, but this difference does not influence their metabolism i.e. activation. It has been reported that both forms on activation exhibit identical reactions in human body (Fieser and Fieser, 1959). Furthermore, investigational studies on animals revealed that D2 is less lethal than D3, yet it is not proven in humans (Ross *et al.*, 2011).

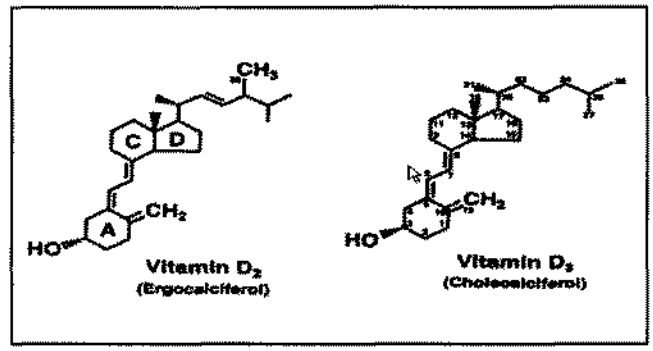


**Figure 1.1**: Structures of vitamin $D_2$ and vitamin $D_3$. (Adapted from Holick *et al.*, 2011)

## 1.2 Sources of Vitamin D

Vitamin D can be attain with the help three main sources in human body.

1. Sunlight
2. Diet
3. Supplements

**1. Sunlight**

Sunlight exposure is the considered as a chief source to naturally attain vitamin D. When skin is exposed to sunlight its solar UVB (ultraviolet B radiation) having wavelength of 290-315nm, enters into the skin and 7-dehydrocholesterol (precursor molecule of cholesterol) transformed into previtamin D3 which further change into vitamin D3. As this vitamin is related to sunlight so vitamin D is also known as sunshine vitamin. Excessive sunlight exposure is not involve in intoxication of vitamin D3 because vitamin D also go on its own destruction (Holick, 2006). Vitamin D synthesis via diet or supplements does not stay for long time in the blood while the cutaneous production of vitamin D sustain at least two-fold long in the blood (Haddad *et al.,* 1993). For example, a person in bathing suit when open to least erythemal dose of ultra violet radiation compared with the person ingested the dose between 10,000-25,000 IU, his quantity of vitamin D production is similar (Holick & Chen, 2008). However, vitamin D production via skin can be diminish due to number of factors like aging, increase in pigmentation of skin, use of sunblock, time of year or time of day etc. These all aspects intensely effect the cutaneous vitamin D production. Therefore, one can believe that skin's production of vitamin D is quite low during the winters especially in European countries (Holick, 2007; Holick & Chen, 2008).

**2. Diet or Food**

Vitamins D2 and D3 intake are absorbed in small intestine which is then integrated with chylomicrons and lipoprotein to move towards liver (D Carter, 2011). As far as diet is concern,

the number of naturally vitamin D enriched food is relatively low. For example oil of fish liver (cod liver oil), oil-rich fish/fatty fish (salmon, trout, and mackerel), and shiitake mushrooms naturally encompass highest aggregate of vitamin D, while a very slight quantity of vitamin D exist in liver of beef, egg yolks and cheese (Ovesen *et al.,* 2003). Similarly, there are a small number of foods that are found to be fortified with vitamin D like fortified butter, milk, yogurt, margarine, orange juices, breakfast cereals etc. In Canada and United State fortified milk with vitamin D is used but most of the countries in Europe banned the nutritive fortification by vitamin D because in one report of 1950, intoxication of vitamin D in adults and children had been observed. Even though, vitamin D is now added to breads, cereals and margarine in various European countries (Holick, 2007).

**3. Supplements**

Food fortification as well as in vitamin D supplements both forms i.e. vitamin D2 and vitamin D3 are employed (Holick, 2007). Although there is availability of two forms of vitamin D supplements but in general, vitamin D3 is endorsed over vitamin D2 and it exhibit huge and more sustained amount in 25hydroxyvitamin D levels in blood. International Units (IU) and micrograms (μg) are the units used to measure vitamin D.

1μg = 40 International Unit of vitamin D2 or D3 (Heaney *et al.,* 2010).

## 1.3 Pathway Involved in Absorption of Vitamin D

Vitamin D that acquires from either sources i.e. diet or skin, both are biologically inactive and entails further hydroxylation to get the active product. The first compartment of the vitamin D in human body is liver where first hydroxylation process takes place to become 25-hydroxyvitamin D (25(OH)D). This addition of oxygen and hydrogen molecule is done by an enzyme named 25-hydroxylase (25-OHase). 25-hydroxyvitamin D then travel towards the kidney where it acquires a pair of $H_2$ and $O_2$ molecules to establish its biologically active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) and this transformation is done via an enzyme 1α-

hydroxylase (CYP27B1). CYP27B1 stands for "Cytochrome P450, Family 27, Subfamily B and Polypeptide 1" is a gene involve in synthesis of 1 α-hydroxylase and that of $1,25(OH)_2D$ (Holick *et al.*, 2011). Expression of 1α-hydroxylase gene is known in several extra-renal tissues, but its involvement in synthesis of $1,25(OH)_2D$ is unidentified in these tissues (Ross *et al.*, 2011). Furthermore, $1,25(OH)_2D$ within the kidneys, small intestine and other target tissues binds to receptor molecule of vitamin D i.e. VDR (Holick *et al.*, 2011), heterodimers were formed with retinoid X receptor (RXR) and recruiting further cofactors that are responsible for transcription in order to regulate the transcription of target gene comprising genes of cell differentiation, cell proliferation and cell apoptosis (Deeb *et al.*, 2007).

$1,25(OH)_2D$ is involve in variety of biotic actions, like persuading and hindering terminal differentiation and proliferation of cells respectively, stimulates production of insulin, inhibiting angiogenesis and production of renin (Liu *et al.*, 2006; Adams & Hewison, 2010). Absorption of intestinal calcium and phosphorus is stimulated by active molecule 1,25 dihydroxyvitamin D (Christakos *et al.*, 2003). The quantity of calcium and phosphorus absorption is affected by the absence of vitamin D i.e. only 10-15 percent and 60 percent approximately of calcium and phosphorus, respectively, are absorbed when there is no vitamin D in human body. Therefore, absorption of intestinal $Ca^{+2}$ and P is enhanced by 30–40% and 80%, respectively, when vitamin D is available in sufficient amount (Heaney, 2004; Holick, 2007).

Apart from cellular concentration of $1,25(OH)_2D$, vitamin D can be degraded by itself by bringing another enzyme's expression named 24,25 $(OH)_2D_3$ (25-hydroxyvitamin D-24-hydroxylase) which is coded by CYP24A1 and it involves in the catabolism of both 25(OH)D and $1,25(OH)_2D$ and transform into biological inert, water soluble calcitroic acid. In catabolism process hydroxylation of $1,25(OH)_2D$ at position C24 is done which convert it into 1,24,25D.

The biological inert product is eliminated via kidney into the urine (DeLuca, 2004; Liu *et al.*, 2006). Schematic representation of vitamin D absorption is shown in figure 1.2.

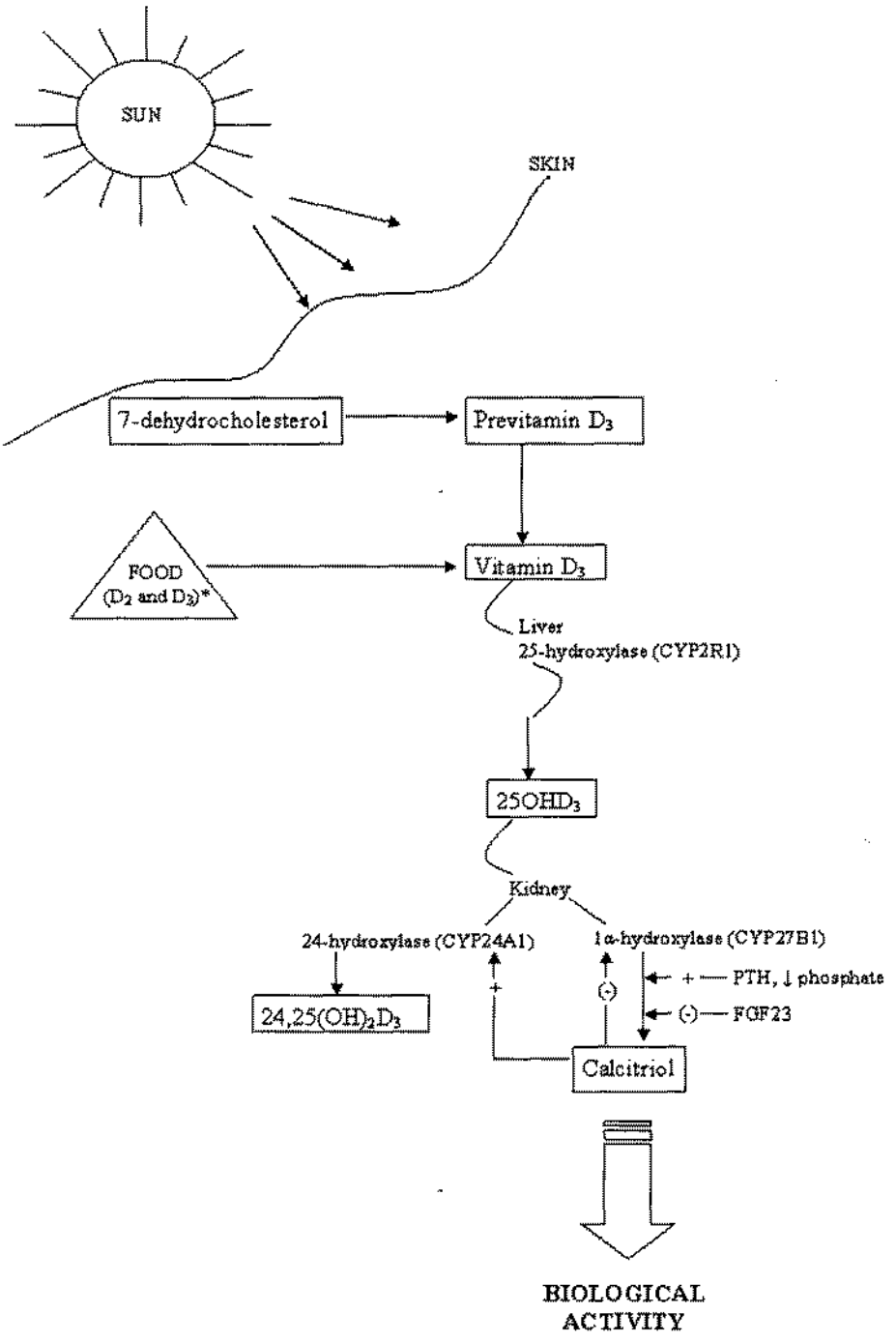


**Figure 1.2:** An overview of pathway involved in vitamin D absorption and activation

(Adapted from Del Valle *et al.*, 2011).

## 1.4 Causes of Vitamin D Deficiency

As a matter of fact that natural sunlight is considered to be the foremost source of vitamin D, therefore the primary reason behind the vitamin D deficiency is insufficient sunlight exposure (Holick & Chen, 2008). Use of sunblock as a skin protector reduces the cutaneous synthesis of vitamin D greater than the 95 percent. Individuals having dark skin colour naturally have ability to protect from sun therefore requiring 3-5 times more sunlight exposure in order to synthesize the equal amount of vitamin D as compared to individuals having white skin tone (Clemens *et al.*, 1982; Hintzpeter *et al.*, 2008). There are number of reasons which are involve in deficiency of vitamin D. Obesity is also associated with vitamin D deficiency, as serum 25-(OH) D and body mass index (BMI) exhibit an inverse association for example rise of $1kg/m^2$ in BMI leads towards reduction of 1.15 percent in 25-hydroxtvitamin D levels (Wortsman *et al.*, 2000). Hence, weight loss or reduction of weight may enhance the vitamin D level. In addition, patients of nephrotic syndrome fail to maintain binding of 25-hydroxyvitamin D and DBP in the urine. Similarly, patients of fat mal-absorption syndromes sometimes incapable of absorbing fat soluble vitamin D (Holick, 2007). The usage of large number of medicine including anticonvulsants and medicine used on AIDS/HIV treatment boost the catabolism mechanism of 25(OH)D and 1,25(OH)2 D (Zhou *et al.*,2006).

It is astonishing to realize the fact that vitamin D deficient cases are very vast even in a sunshine country where there is an existence of immense amount of sunlight. This might be due to the reason that either sufficient amount of vitamin D is not absorbed in our body or the defined optimal values should be revised (Khan *et al.*, 2013). Vitamin D deficiency can be initiated owing to the number of external reasons, for an instance, generally women do not prefer to go outside the home or intricate in domestic work, sun block usage, females wearing hijab exposing only hands or face, increased pigmentation owing to which extensive amount of sunlight exposure is necessary. Considering the above elements, this cannot be applied to

sunniest areas like South America where dressing style might not create the hurdle in sunlight absorption but still existence of deficiency of vitamin D becoming an important health issue (Holick & Chen, 2008; Khan *et al.*, 2013).

## 1.5 Consequences of Vitamin D Deficiency

Consequences of prolonged vitamin D deficiency in adults comprises weakness of muscles, osteomalacia, osteoporosis and increased risk of falls (Janssen *et al.*, 2002; Bischoff-Ferrari *et al.*, 2005). Intake of vitamin D in reduce amount and presence of less amount of vit. D metabolites in blood, both are related with increased incidence and severity of numerous autoimmune diseases including T helper type 1 lymphocyte (Adorini *et al.*, 2008; Zold *et al.*, 2008), psoriasis (Tanghetti, 2009), type I diabetes (Mohr *et al.*, 2008), multiple sclerosis (Munger *et al.*, 2006) and rheumatoid arthritis (Patel *et al.*, 2007). Similarly effect of vitamin D deficiency is the irregularities in bone, calcium and phosphorous metabolism. Moreover, owing to the deficiency of vitamin D the efficiency of $Ca^{+2}$ and P absorption in intestine reduces, which in turn increased the parathyroid hormone levels.

The parameter which maintains serum calcium within its normal range is secondary hyperparathyroidism. It is responsible for two things:

1. Mobilizing calcium from the skeleton.
2. Increase phosphorous progressive in the kidneys.

The local foci of weakness in bone is occurred due to increase in the parathyroid hormone mediated osteoclastic activities. Due to this increase, a generalised decrease in BMD (bone mineral density) is occurred which results in osteoporosis and osteopenia.

The presence of low amount of minerals in skeleton of young children may cause variation in skeletal abnormalities which referred as rickets. Vitamin D dependent rickets is characterized hypocalcemia and hypophosphatemia in blood and this disorder results in soft and weak bones which leads towards fractures. Individuals having rickets are with bowed legs

(Thacher & Fischer, 2009). On comparison to children, sufficient amount of mineral is present in the adult's skeleton in order to protect the deformities of skeleton, therefore osteomalacia which is known to be deficiency of mineralization, frequently goes unnoticeable. However, reduction in bone mineral density is owing to the osteomalacia and generalized or isolated muscles and bone pains are associated to osteomalacia (Plotnikoff & Quigley, 2003). Deficiency of vitamin D also involve in weakening of muscles i.e. children having vitamin D deficiency may face problem in walking and standing (Gordon *et al.*,2008), while the old individuals have more frequent falls, thus risk of fractures may increases (Bischoff-Ferrari *et al.*,2009).

Vitamin D endocrine system comprises of seco-steriod hormone vitamin D itself, its receptors and the metabolising enzymes, and this endocrine system of vitamin D is known to be majorly involve in skeleton metabolism. In addition, literature also shows that vitamin D endocrine system also appears to be a significant element in other metabolic pathways, for example, those comprising of immune response and cancer. Immune system taking as an example, vitamin D is responsible to stimulate the differentiation of monocyte and hinders cytokines secretion and proliferation of lymphocyte, for an instance interleukin-2, interleukin-12 and interferon-γ. Furthermore, vitamin D also inhibit cell proliferation among variety of cancerous cells (Haussler *et al.*, 1998).

## 1.6 Prevalence of Vitamin D Deficiency World Wide

As level of Vitamin D is acquire by measuring 25-hydroxyvitamin D in blood, therefore different categories according to the blood levels have been recommended. Deficiency of vitamin D is referred to <20 ng/ml (50 nmol/liter) of 25-hydroxyvitaminD. Similarly 21-29 ng/ml (525–725 nmol/liter) of 25-hydroxyvitaminD is considered its insufficient amount and above 30 ng/ml is sufficient amount of 25-hydroxyvitamin D in blood. At the same time,

presence of an excessive amount of vitamin D (greater than 150 ng/ml) in blood might cause vitamin D intoxication (Michael *et al.,* 2011).

Considering above definitions of vitamin D levels, probably 1 billion people either have insufficient amount of vitamin D or they are vitamin D deficient. According to number of reports it has been observed that 20-100% older women and men who are still part of Euorpean, Canadian, and U.S. community are suffering from deficiency of vitamin D (Lips *et al.,* 2006; Holick, 2006; Holick, 2007). Deficiency of vit. D is commonly observed in the Middle East, India, Africa, Australia and South America. High risk for deficiency and insufficiency of vit. D is not age specific but it is equally subjected in all ages worldwide, meaning thereby from children to young one and also adults of middle-aged (Marwaha *et al.,* 2005; Thacher *et al.,* 2006). For an instance, study conducted in Boston investigated that African-American and Hispanic youngsters with percentage of 52 and in Maine white preadolescent females with the percentage 48 exhibited less than 20 ng/ml level of 25-(OH)D (Sullivan *et al.,* 2005). According to another report, 42 percent of black preadolescent girls and women with age of 15 to 49 years all over the United State possess very low level of vitamin D i.e. below 15 ng per milli-liter at the completion of winter season (Nesby-O'Dell *et al.,* 2002), similarly there are 32 percent students with good health conditions and physicians at Boston hospital which possess vitamin D level less than 20 ng/ml (Tangpricha *et al.,* 2002). Furthermore, suboptimal level of 25-hydroxyvitamin D is found among at least 50% of postmenopausal women that are on osteoporosis treatment and this suboptimal level is < 30 ng/ml (Holicks *et al.,* 2005; Lips *et al.,* 2006).

As in Europe countries small number of vitamin D fortified foods are present, so all age group individuals are found to be on high risk. Individuals having good sunlight exposure exhibit elevated levels of vitamin D i.e. >30 ng/ml. On the other hand, deficiency of vitamin D is also very common even in the sunny countries when the large skin area is protected from

sun. For an instance, 30-50 percent of children as well as adults exhibited below 20 ng/ml of vitamin D in Australia, the United Arab Emirates, Saudi Arabia, India, Lebanon and Turkey (Sedrani, 1984; McGrath *et al.*, 2001). According to one study in Australia it was investigated that one in three individuals showed vitamin D deficiency (Daly *et al.*, 2012). Apart from all above circumstances, women expecting a baby and lactating women were also on high risk for deficiency of vitamin D despite the fact that they are taking prenatal multivitamins as well as supplements of vitamin D. It has been estimated that 93 and 90 percent pregnant women intake approx. 2.3 glasses of milk and fish respectively per day and 70 percent acquired a prenatal vitamin (Hollis & Wagner, 2004; Bodnar *et al.*, 2007; Lee *et al.*, 2007).

Inadequate sunlight exposure and poor oral intake of vitamin D is also associated to vitamin D deficiency rickets. Though in developing countries the use of fortified milk having vitamin D supplements and the sufficient sunlight exposure are provoked but still vitamin D deficiency rickets has potential to occur on large scale population in children, like in China. Literature showed that 26.7% children had rickets among rural Chinese (Strand *et al.*, 2007).

## 1.7 Prevalence of Vitamin D Deficiency in Pakistan

Other than United States, Europe, Saudi Arabia, Turkey, China, Australia etc., huge number of vitamin D deficient individuals have been reported in Pakistan as well. Number of surveys which is conducted in past few years indicated that there is 85% non-pregnant and pregnant mothers having deficiency of vitamin D. 41.1 percent deficiency of vitamin D among children had been reported in Pakistan comprising 31% and 10.1% as deficient and severe cases respectively. As far as levels of vitamin D is concerned it is estimated in one study of Faisalabad that 77.5% and 18% individuals were vitamin D deficient and insufficient respectively (Masood *et al.*, 2012). Females in another study of Faisalabad revealed the elevated prevalence of vitamin D deficiency. 10 and 87% pregnant women exhibited insufficiency and deficiency of vitamin D respectively while 3 percent pregnant women exhibited normal level of vitamin

D (Aslam *et al.*, 2012). Similarly, research results from Karachi revealed that three hundred and five premenopausal females showed vitamin D deficiency with the percentage of 90.1 (Khan *et al.*, 2012).

## 1.8 Genes Involved in Synthesis of Vitamin D

In order to understand the variances in vit. D endocrine system (vitamin D, its receptor and metabolizing enzymes) among variety of individuals, research needed to focus on the effect of modifications that is carried out in DNA sequence of various protein of this endocrine system. The cytochrome P450, family 2, subfamily R, polypeptide 1 (CYP2R1), CYP27B1, group specific component (GC), 7-dehydrocholesterol reductase/nicotinamide-adenine dinucleotide synthetase 1 (DHCR7/NADSYN1), CYP24A1, vitamin D receptor gene (VDR) and CYP27A1 these all are the genes that intricate in metabolism and transportation of vitamin D (White & Cooke, 2000; Cheng *et al.*, 2003). The GC is involved in transportation of vitamin D and, CYP27B1, CYP2R1 and CYP24A1 responsible for hydroxylation and de-hydroxylation. Moreover NADSYN1 is well-known to be involved to modulate vitamin D (Ahn *et al.*, 2010). Below are the details of these genes.

Vitamin D3 is naturally synthesize in the skin and subsequently number of biological process, the active product $1,25(OH)_2D_3$ is synthesised in the liver. 1,25dihydroxyvitamin D3 synthesis is accomplished by the gene named CYP27B1 which is located on chromosome 12q13.1–q13.3, which encode an enzyme named 25-hydroxy-vitamin D-1 alpha hydroxylase. So, genetically the vitamin D level is dependent on CYP27B1 gene as it gives the bioactive form of vit. D and variants of this gene can be studied in order to investigate its association with other diseases (Sundqvist *et al.*, 2010). A range of CYP27B1 variants is involve in the association of number of disorders such as type 1 diabetes, multiple secrosis etc. (Bailey *et al.*, 2007).

The group specific component (GC) gene belongs to family albumin, comprises transportation of vitamin D and it is located on 4q12-q13. GC encoded single chain polypeptide that consists of 474 amino acids residues (Speeckaert *et al.*, 2006).

A glutamine-dependent enzyme named nicotinamide adenine dinucleotide synthetase 1 (NADSYN1) is coded by NADSYN1 gene and NADSYN1 enzyme is responsible for production of cholesterol by converting 7-dehydrocholesterol into cholesterol and helps in the synthesis of coenzyme named nicotinamide adenine dinucleotide. This coenzyme is essential for synthesis of lipid and production of energy (Bu *et al.*, 2010). Gene locus of NADSYN1 is on chromosome 11q13.4 (nearby dehydrocholesterol reductase gene). Genome wide association study demonstrated that polymorphism in the genes of cholesterol production has great impact on vitamin D status (Wang *et al.*, 2010). The risk of inadequate amount of vitamin D and of dyslipidemia showing an association with SNP rs12785878 of this gene in the literature (Foucan *et al.*, 2013). A syndrome named Smith-Lemli-Opitz Syndrome is linked with the changes that occur in dehydrocholesterol reductase gene (DHCR7) sequence and it is characterized as homozygous individual exhibit low level of cholesterol in serum (Wang *et al.*, 2010).

Furthermore, very rare monogenetic disease named 1,25 di-hydroxyvitamin D resistant rickets occurred owing to the lethal mutations in VDR gene. On the other hand, polymorphism in vitamin D receptor gene frequently arise in the population but the scientifically analysis have not done yet and the effects of polymorphism on VDR function are not clearly understandable. Consequences of this polymorphism on vitamin D metabolic pathway along with complicated disorders like osteoporosis are under consideration (Lander & Schork, 1994; Risch & Merikangas, 1996). The presence of anonymous RFLP in VDR (vitamin D receptor gene) is creating hurdle in elucidating the polymorphism in VDR gene. Therefore, in order to locate the genes residing in the area, one need to be investigate the locus of the vitamin D receptor gene

which helps in understanding the association mechanism. In addition, all related polymorphisms in VDR are then divided in groups and at the end their association with currently used markers of restriction fragment length polymorphism (RFLP) can be determined (Uitterlinden *et al.*, 2004).

Vitamin D deficiency is risk factor for causing dementia as its function in brain is clear (McCann *et al.*, 2008). NOTCH3 is one of the gene which involve in causing dementia (Joutel *et al.*, 1997). NOTCH3 is highly conserved trans-membrane receptor whose pathway plays a central role in muscle development and regeneration (Schuster-Gossler *et al.*, 2007; Buas & Kadesch, 2010). The location of NOTCH3 gene is on chromosome 19q12 (Joutel *et al.*, 1996).

## 1.9 Aim of the Study

Deficiency of Vitamin D is consider to be top most common nutrition responsive medical condition in the world. That is why number of scientific publications on vitamin D has surpassed publication on any other vitamins for 21$^{st}$ century (Glade, 2012). The amount of vitamin D inadequacy has been calculated which is approx. 1 billion worldwide. There is a high prevalence of deficiency in Bangladesh, Pakistan, Middle Eastern countries, Iran, and India, (Hossain *et al.*, 2011). Vitamin D status in Pakistani population is not well documented. There are few reports available involving studies with small sample size of few cities. For example, pregnant women with percentage of 89 were vitamin D deficient in Karachi with a vitamin $D_3$ serum levels below 30 ng/mL (Hossain *et al.*, 2011). Another report suggested 90% of adults and apparently healthy volunteers were vitamin D deficient (Mansoor *et al.*, 2010).

Keeping in view the alarming situations it is need to considered the bioavailability of vitamin D and the factors hindering its production. Two genes were selected for the present study, CYP27B1 and NOTCH3 gene. Dementia is known to be associated with vitamin D deficiency and NOCTH3 gene is not directly involve in vitamin D pathway but involve in causing dementia, so in relation to dementia link with vitamin D deficiency NOTCH3 gene

was selected to correlated the relation of NOTCH3 gene with vitamin D deficiency. D19S415 and D19S714 markers of NOTCH3 gene were used in order to find out the linkage with the vitamin D deficiency in selected vitamin D deficient family. Secondly, CYP27B1 was selected because it was involved in production of biological active product of vitamin D i.e. 1, $25(OH)_2$ D, so this active product was the main focus. SNP rs 10877012 of CYP27B1 gene was used for genotyping on cases samples of vitamin D deficient patients. Along with genetic etiology, clinical history of vitamin D deficient patients was statistically analysed.

# MATERIALS AND METHODS

# 2 Materials and Methods

The present study comprises on two sections i.e. clinical assessment of vitamin D deficient patients with their statistical analysis and the genetic etiology of the vitamin D deficiency. The research was conducted in Institute of Biomedical and Genetic Engineering, Islamabad. The only inclusion criteria of present study was the patients with low vitamin D level or exhibited the history of vitamin D deficiency regardless of age or gender. There was neither age limit for the individuals nor was it the gender defined study.

## 2.1 Data Collection

The study comprises of 523 patients with vitamin D deficiency including 430 females and 93 males of different socioeconomic status. In addition, 41 blood samples of single family who showed vitamin D deficiency and multiple disorders were also collected. Clinical history of patients along with their blood samples were collected. A Performa was made for this purpose and variables like name, age, gender, caste, address, vitamin D levels, history of vitamin D associated problems, onset of vitamin D deficiency, syndrome manifesting vitamin D deficiency etc. were covered in patient's history. The collected information of patients was then documented for further analysis.

## 2.2 Blood Sample Collection

Blood samples of patient with low level of vitamin D were acquired in the present study. At the same time blood samples of those patients which had previous record of vitamin D deficiency but recovered after medication, were also collected. Sterile syringes were utilized to draw the venous blood sample in EDTA containing vacutainers. The required blood volume was 5cc-10cc per each sample. The blood sample of these patients were collected from different hospitals of Pakistan like Holy Family, Fuji Foundation, POF, KRL etc. Blood samples were then stored at 4°C till further processing.

## 2.3 Allocation of Identity Number to Each Sample

The collected blood samples were then given an identity numbers in order to use them in further process accordingly. The identity numbers were like VD 01, VD 02, and VD 03 and so on.

## 2.4 Extraction of Genomic DNA

After the allotment of identities to blood samples, the very next step was the extraction of genomic DNA. The protocol used for DNA extraction is given below.

### 2.4.1 DNA Extraction Protocol

DNA extraction were accomplished via phenol extraction method or organic method. The chemicals or reagents that were used in this process are listed in table 2.1. The extraction method was finalized in three days and steps involved in this protocol are as followed:

**DAY-1**

1. Blood sample were transferred into the falcon tubes (50ml). Measured their volume and to the blood sample added three times of cell lysis buffer. All tubes were shaked well and keep them on ice for 30 min.
2. Centrifuged the tubes after 30 min at 1200 rpm for 10min at 4°C.
3. Supernatant was discarded and re-suspended the pellet. 15ml more cell lysis buffer were added in each sample. Tubes were again centrifuged at 1200 rpm for 10min at 4°C.
4. Supernatant was discarded and pellet was re-suspended.
5. 5ml of STE was added and 250ul of SDS were added drop wise while vortex.
6. At the end of the Day 1st 10 ul of proteinase K (20 mg/ml) was added in each sample. Placed the samples in water-bath at 55 °C for overnight.

**DAY-2**

1. Next day 5ml of equilibrated phenol (pH=8) was added in each sample and tubes were shaked for 10 min and afterwards keep on ice for 10min. Centrifuged at 3200rpm for 30min at 4°C. By the addition of phenol, organic and aqueous layer were separated. The upper aqueous layer of each sample was removed with cut tips into separate labelled falcon tubes.
2. 5ml chilled chloroform-isoamylalcohol (24:1) was added. Same procedure of shaking was repeated for 10 minutes and placed on ice for 10 min. Spin at 3200rpm for 30min at 4°C. The upper aqueous layers of each samples were again removed with cut tips into separate labelled falcon tubes.
3. In the aqueous layer 500 ul of 10M ammonium acetate and 5ml of chilled Isopropanol (or 10ml of chilled absolute ethanol) were added and shaking of tubes was done until DNA precipitated out as visible white threads. Samples were placed at -20°C for overnight.

**DAY-3**

1. Samples were centrifuged at 3200rpm in Centrifuge (Eppendorf 5810 R) for 60min at 4°C, after that discarded the supernatant & re-suspended the pellet.
2. 5ml of chilled 70% ethanol was added and then centrifuged for 3200 rpm for 40min at 4°C.
3. Supernatant was discarded and by placing the falcon tubes inverted pellet was desiccated. Next day re-suspended in 10Mm Tris-HCL (pH8.0, with 500ul volume of Tris added).

**Table 2.1: Composition of Reagents Used in DNA Extraction**

| Reagents | Chemicals With Their Compositions |
|---|---|
| Cell Lysis Buffer | 1. KHCO3 (Potassium Bicarbonate) =1 gm/L<br>2. $NH_4Cl$ (Ammonium Chloride) =8.29gm/L<br>3. 0.5M EDTA (Ethylene Diamine Tetra Acetate) =200µL<br>Volume was measured accurately and made up to 1L using $dH_2O$. Stir it on magnetic stirrer and then stored at room temperature. |
| STE (Saline Tris EDTA) | 1. 3M NaCl (Sodium chloride)= 33.3ml<br>2. 1M Tris –HCl (pH=8)=4.0ml<br>3. 0.5M EDTA (pH=8) = 2.0ml.<br>Volume was measured accurately and made up to 1L using $dH_2O$. Stored at room temperature |
| SDS(Sodium Dodecyl Sulfate )Solution | 1. SDS = 10gm (10%)<br>Reagent was mixed and volume was made up to 100 ml with $dH_2O$ filtered with 0.4mm filter paper. Stored at room temperature. |
| Proteinase k (20mg/mL) | Stored at -20°C till further use. |
| 1M Tris | Trisma Base= 121.1g<br>For 2 Liters=121.1*2=242.2g<br>Set pH=8 |
| Chloroform-Isoamyl Alcohol (24:1) | 1. Chloroform=480ml<br>2. Isoamylalcohol=20ml<br>Above two solutions were mixed in a glass cylinder under the hood and stored at 4°C. |
| Isopropanol and Ethanol | Stored at room temperature and was ready to use. |

## 2.5 Qualification and Quantification of Isolated DNA Stock

DNA samples or DNA stock were quantified by Optical Density (OD) measurement.

### 2.5.1 Optical Density Measurement

Optical density was used to measure the DNA concentrations among DNA stock via NanodropTM spectrophotometer. The concentration of nucleic acid lies between the wavelengths of 260nm and 280nm. DNA samples with impurities like proteins or traces of proteins will show OD value less than this range i.e. 260-280 nm wavelength. Take optical density (OD) of the samples at 260 nm & 280 nm. (Ideally 260/280 ratio=1.7-2.0, Ratio>2.0 phenol contamination; Ratio<1.7s protein contamination). Meaning thereby, presence of protein contamination indicate that estimates of DNA concentration would be inaccurate (Glasel, 1995).

DNA concentration is calculated by following formula.

Abs 260nm × 50 = DNA concentration (ug/ml)

(Where 50 is correction factor)

### 2.5.2 Dilutions of DNA Stock

As DNA stock was very concentrated so working solution was made by diluting the DNA stock. The OD values was used in dilution ratio i.e. according to OD values of each sample, ratio of $dH_2O$ was decided. Higher the OD value higher was the amount of $dH_2O$.

## 2.6 Primer Designing

With the help of UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway), primers were designed for the selective STS markers of NOTCH 3 gene and also for the SNP of CYP27B1 gene (rs 10877102). Primers along with their genes are listed below in the table 2.2. The efficacy of these designed primers was checked against human genome by using the computational tool i.e. In-Silico PCR which can be access online via

http://genome.ucsc.edu/cgi-bin/hgPcr?command=start. At last the selected STS and SNP markers were synthesized by Integrated DNA Technologies (IDT).

**Table 2.2: List of Primers**

| Gene Name | Marker Name | Left Primer | Right Primer | Product Size |
|---|---|---|---|---|
| **NOTCH 3** | D19S415 | GAT GTT GTT GCA TTG GC (17) | GGG TCA TGG TAT GCT CC (17) | 250 bps |
| **NOTCH 3** | D19S714 | ATG CCC TCT TCT GTC TCT CC | GCA GAG AAT CTG GAC ATG CT | 250 bps |
| **CYP27B1** | rs 10877012 | GAG GCT AAG GTG GGC AGA (18bps) | ATG CCT TCT ACC ATC CTC CT (20bps) | 200-250 bps |

## 2.7 Polymerase Chain Reaction (PCR) Analysis

Amplification of the cases samples as well as family samples of vitamin D deficiency was accomplished via PCR. Firstly, PCR was for the vitamin D deficiency family with the both markers (D19S415 and D19S714) and, secondly, it was for all collected cases samples of vitamin D deficiency. Results of the corresponding markers are shown in Results section of this study.

### 2.7.1 Preparation Method for PCR

Polymerase chain reaction was executed in 2 ml eppendorf tubes. These 2ml tubes having total reaction mixture of 25µl including 3µl diluted DNA and 22µl master mixture in it. The master mixture was prepared and centrifuged at short spin to mix the reagents gently. The ingredients or reagents used to make master mixture is enlisted below in table 2.3 along with their concentrations. PCR was performed on 25µl reaction mixture comprising 2µl of each Taq Buffer, dNTPs and magnesium chloride, 3µl genomic DNA, 0.5µl of each primers, 0.4µl Taq polymerase and deionized water to

make up the volume according to the required reactions. The process of this thermo cycling was accomplished in Thermo Electron Corporation (PxE 0.2 Thermo Cycler).

### 2.7.2 PCR Cycles

The temperature profile or the programing of thermo cycler entails the initial/pre denaturation, annealing, extension and final extension or elongation. PCR cycles along with their temperature and time is shown in table 2.4.

**Table 2.3: PCR Reagents and their Concentrations**

| Ingredients | Final Conc. | Stock | Required Volume |
|---|---|---|---|
| Genomic DNA | 50 ng | 25 ng/µl | 3 µl |
| Primer Forward | 0.5µM (5pmol) | 10µM | 0.5 µl |
| Primer Reverse | 0.5µM (5pmol) | 10µM | 0.5 µl |
| dNTPs (dATP,dTTP,dCTP, dGTP) | 0.2mM (200µM) | 2.5 mM | 02 µl |
| PCR Buffer | 1x | 10x* | 02µl |
| Magnesium Chloride | 2.0mM | 50mM | 02 µl |
| Taq Polymerase | 1 units | 5 units/µl | 0.4 µl |
| $dH_2O$ | - | - | **q.s to 25 µl |

* 10X PCR buffer (100 mM Tris Cl-pH 8.4, 500 mM KCl, and 1% Triton)

** q.s = quantity sufficient

**Table 2.4: PCR Cycles**

| Steps | Cycle | Time | Temperature |
|---|---|---|---|
| **Initial denaturation** | 1 x | 04 minutes | 95°C |
| **Denaturation** | | 45 seconds | 94°C |
| **Annealing** | 35 x | 45 seconds | 58°C |
| **Extension** | | 45 seconds | 72°C |
| **Final Extension** | 1 x | 10 minutes | 72°C |

## 2.8 Agarose Gel Electrophoresis

Amplification of the PCR product was confirmed by 2% agarose gel electrophoresis. The protocol used to prepare 2% agarose gel is as follow:

- Assembly of gel caster was set i.e. placing clamps or taping either sides of the gel caster, and adjusted combs in the respective site of the gel caster to make wells for DNA loading.
- Weighed 6gm agarose in order to make 2% gel in 250ml of 1X TBE buffer.
- Agarose was heated with 1X TBE buffer in microwave oven till the time of boiling. Agarose solution prepared.
- Mixed 10 µl EtBR (10 µg/µl) into 250 ml agarose solution when it had temperature of <60°C, poured into gel caster and left the gel for solidification at room temperature or under the fan for at least 30-45 min.
- After solidification combs were removed smoothly and gel was shifted into electrophoresis tank, 1X TBE buffer added in tank to cover the surface of gel up to 2 mm.
- 5 µl from amplified DNA and 5 µl loading dye were mixed in a mixing plate (or in a PCR tube), loaded this mixture into a well within gel alongside a standard DNA sample or ladder (25 ng/µl), electrophoresis tank attached with the electric supply, powered on the electric supply and ran the setup. Estimated run time was 45 min for 200 Volts.
- With the help of trans-illuminator or gel documentation system, separated DNA under UV light was visible and its concentration was estimated by comparing its intensity with standard DNA or the adjacent DNA fragment of ladder/marker.

## 2.9 Linkage Analysis

PCR Product of the selected vitamin D deficient family was further used to check the linkage of D19S415 and D19S714 markers in the selected family. For this purpose polyacrylamide gel electrophoresis was used and haplotypes were made using PAGE results and was further analysed. The protocol used for PAGE is as follow:

### 2.9.1 Protocol for Polyacrylamide Gel Electrophoresis

Prepared 25% Ammonium per Sulphate (APS) solution as follow.

25% APS solution= 1g APS + 4ml $dH_20$

1. **Gel Solution Preparation:**
   - 40% Acrylamide = 75ml
   - 10x TBE Buffer= 25ml + 5ml Glycerol
   - Added $dH_20$ for final volume up to 250ml in plastic cylinder and mixed vigorously.
   - Took 50ml of it in measuring cylinder and shifted rest of it in to beaker.
2. **PAGE Assembly:**
   - Wiped PAGE glass plates with Sigma Silica coat to prevent gel from sticking glass plates.
   - Set the base and glass plate with the separator firmly.
3. **Base Gel (For Quick Polymerization):**
   - Took 50ml from prepared gel solution.
   - Added 300ul TEMED and 300ul 25% APS.
   - Shaked well and poured in base assembly and left for it for 5-10 minutes.
4. **Loading of Gel:**
   - Took 850ul of 25% APS, 150ul TEMED and 200ml gel solution in a beaker.

- Mixed well and poured it between the PAGE plates. Tapped slowly for smooth flow during pouring.
- Removed air bubbles during pouring gel.
- Placed comb and aligned margin of comb with plates.
- Poured a little gel solution on comb.
- Left it for 50-60 minutes for smooth polymerization.
- After 60 minutes, removed base assembly and placed in base tank.
- Poured 1xTBE buffer in buffer tank and bottom of base.
- Fixed electrodes and pre-run gel for 10 minutes at 110 watts. It softens the gel and helped to remove the comb.

5. **Sample Loading:**
   - Added 8ul of 6x loading dye in 8ul of PCR products and mixed them properly.
   - Loaded sample with thin white tips.
   - Run at 110 watts for 2 hours.
   - Checked the results under UV trans-illuminator.

## 2.10 Statistical Analysis

The clinical history of all patients was documented on Microsoft excel sheet and this documented data was further analyse and evaluated with the help of statistical tool named SPSS 20 (IBM SPSS, Inc., USA). By applying different statistical test, data was analysed, frequency of different parameters was calculated. Moreover, cross tabulation between different parameters were also done. The pie charts and histograms were also drawn by SPSS 20. The results of these analysis are shown in chapter 3 named "Results".

# RESULTS

# 3 Results

The vitamin D deficient patients were included in the present study. To evaluate the clinical history of patients via SPSS, firstly, frequency of parameters like gender, age, caste, onset of vitamin D deficiency, range of the vitamin D deficiency levels and other frequencies were calculated and afterwards cross tabulation of two variables/parameters was calculated. Following were the outcomes of present study.

## 3.1 Statistical Analysis

### 3.1.1 Frequency of Parameters



By calculating the gender frequency, results revealed that females were in elevation than males in the present study i.e. among 523 patients 430 were females and 93 were males (Table 3.1). In addition, the patients with age group of 46-55 were more prone towards low vitamin D deficiency level (Figure 3.1). Furthermore, Sheikh (82 out of 523) and Pathan (66 out of 523) caste were more affected as compared to other castes according to present data with percentage of 16 and 13 respectfully. Other castes like Abasi, Awan, Balouch, Gujjar, Butt, Rajpoot, Parachi, Kashmiri, Kiyani, Qureshi, Syed etc. were also present in present data but with percentage which was near to the ground (1%, 3%, 4% of each caste) (Figure 3.2). The mean age was calculated for the age of onset and it was found to be 44 years. Therefore, according to the data of present study 44 years was found to be the potential age which was prone towards vitamin D deficiency and most of the patients were came with the complain of joints pain (specifically in knee and ankle). In addition osteoporosis, arthritis, diabetes mellitus and few with weakness or weight loss were observed among the individuals at the age of onset of vitamin D deficiency. According to present data, the patients in the range of deficiency (<12 ng/ml) exhibited the highest percentage i.e. 42%, afterwards others patients were in insufficiency range i.e. $\geq$ 12-30 ng/ml with the percentage of 40 and very few exhibited the normal range i.e. >30 ng/ml (Table 3.2). Apart from these parameters other variables like

quality of diet, physical activity, BMI, marital status consanguineous/non-consanguineous marriages and exposure to sunlight were also analysed. It was observed that quality of diet and BMI of patients were normal in most of the patients, however, those who were married and in consanguineous marriages showed the elevated percentage as compared to those who were single and in non-consanguineous marriages respectively. Moreover, there were 53.3% and 45.7% patients who had normal and poor sunlight exposure respectively (Table 3.3).

**Table 3.1: Frequency Table of Gender**

| | No. of Patients | Percent |
|---|---|---|
| **Female** | 430 | 82.2 |
| **Male** | 93 | 17.8 |
| **Total** | 523 | 100.0 |

The frequency table of gender shows that females are present in elevation (82%) than males (Highlighted row represents the females count).

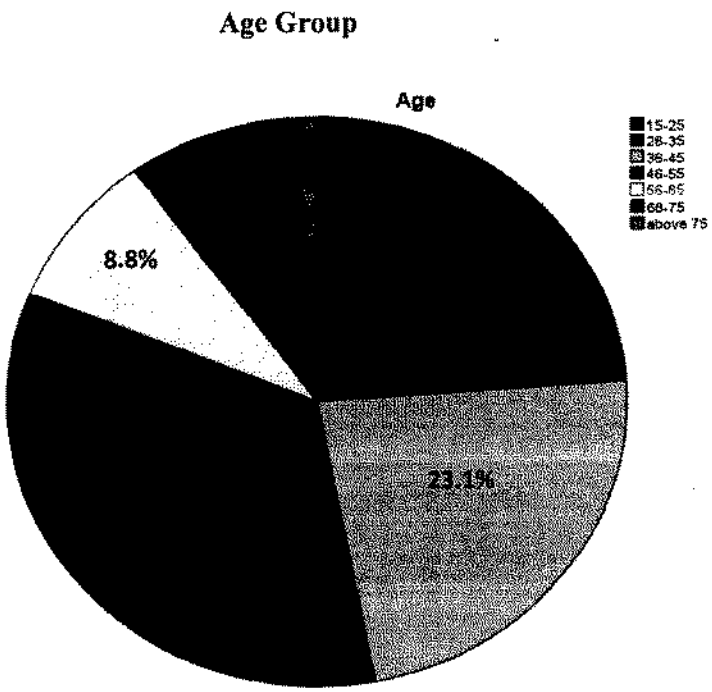


**Figure 3.1:** Pie Chart for Frequency of Age Group. The above pie chart shows that patients belong to the age group 46-55 with 34.4 % are more prone towards low deficiency level.

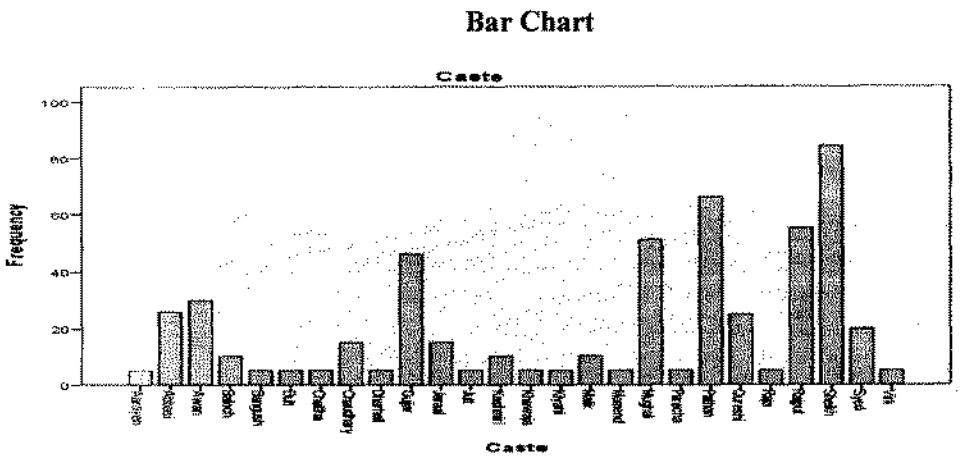


**Figure 3.2:** Bar Chart for Frequency of Caste. The above bar chart indicated Sheikh and Pathan are more affected and after that Rajput and Mughal Caste is in a queue.

**Table 3.2: Frequency Table for the Range of Vitamin D Level**

| Range of Vitamin D Levels | No. of Patients | Percent |
|---|---|---|
| Deficiency(<12ng/ml) | 219 | 41.9 |
| Insufficiency (=12-<30 ng/ml) | 209 | 40.0 |
| Normal (>30 ng/ml) | 95 | 18.2 |

The above table demonstrates that 41.9% and 40% patients' exhibit deficiency and insufficiency levels respectively and small number of patients exhibit the normal range (18%).

**Table 3.3: Frequency Table of Sunlight Exposure**

| | No. of Patients | Percent |
|---|---|---|
| **Good** | 5 | 1.0 |
| **Normal** | 279 | 53.3 |
| **Poor** | 239 | 45.7 |

This frequency table indicates that only 1% patients have good exposure to sunlight and 45.7% have poor sunlight exposure.

### 3.1.2 Cross tabulation of Variables

After analysing the frequency rate of number of variables cross tabulation or comparison of two variables were also analysed using SPSS (Statistical tool). The outcomes of these comparisons are as follow.

#### 3.1.2.1 Age and Gender

Ages of the patients were divided in number of groups with defined age ranges. Results of SPSS analysis elucidated that 55 females and 10 males were vitamin D deficient in the age group of 15-25years. Similarly 48 females and 12 males were deficient from the age 26 years to 35 years. 86 females and 35 males in age group of 36-45years had vitamin D deficiency. The highest count of females was observed in the age group 46-55years and only 26 males were found in this age group. Furthermore, 41 and 42 females came under the age group of 56-65 and 66-75 respectively who had vitamin D deficiency and there were only 5 males in these age groups. Therefore, these results indicated that in every age group the ratio of females was high than the males (Table 3.4).

#### 3.1.2.3 Vitamin D Supplement and Range of Vitamin D Level

To check the response to therapy, vitamin D supplement usage was compared with the vitamin D deficiency levels. Among 523 patients, there was very less amount of patients who were on vitamin D therapy and those who did not use vitamin D supplements exhibited very high percentage. Furthermore, analysis of present data indicated that the highest count of non-vitamin D therapy patients lies under the deficiency range (<12 ng/ml) i.e. total of 293 patients 146 were not using vitamin D supplements and were vitamin D deficient (Table 3.5).

### 3.1.2.4 Gender and Range of Vitamin D Level

After comparing gender with the levels of vitamin D deficiency, results revealed that there was huge difference between the ratio of females and males who had deficiency or insufficient amount of vitamin D. Total of 219 patients of deficiency range (<12 ng/ml) 199 were females and only 20 were males. In addition, the total of 209 patients which came under the range of insufficiency (≥ 12-30 ng/ml), 153 were females and 56 were males. The rest of the subjects were normal encompassing 78 females and 17 males. Therefore, female's ratio was in elevation as compared to males in each range (Table 3.6).

**Table 3.4: Cross tabulation of Age and Gender**

| | | Gender | | Total |
|---|---|---|---|---|
| | | Female | Male | |
| **Age** | 15-25 | 55 | 10 | 65 |
| | 26-35 | 48 | 12 | 60 |
| | 36-45 | 86 | 35 | 121 |
| | 46-55 | 153 | 26 | 179 |
| | 56-65 | 41 | 5 | 46 |
| | 66-75 | 42 | 5 | 47 |
| | above 75 | 5 | 0 | 5 |

This table indicates that females are more affected in every age group (highlighted column shows the female counts). Similarly, females with the age 46-55 years are showing the highest figure.

**Table 3.5: Cross tabulation of Range of Vitamin D Level and Vitamin D Supplement**

| | | Vitamin D Supplement | | | |
|---|---|---|---|---|---|
| | | No Taking | On and Off Taking | Taking Regular | Total |
| Range | Deficiency (<12ng/ml) | 146 | 10 | 63 | 219 |
| | Insufficiency (=12-<30 ng/ml) | 117 | 5 | 87 | 209 |
| | Normal (>30ng/ml) | 30 | 10 | 55 | 95 |
| | Total | 293 | 25 | 205 | 523 |

This table demonstrates that the highest number of patients who do not use vitamin D supplements lies under the deficiency range (Highlighted).

**Table 3.6: Cross Tabulation of Gender and Range of Vitamin D Level**

| | | Range of Vitamin D Level | | | |
|---|---|---|---|---|---|
| | | Deficiency | Insufficiency | Normal | Total |
| Gender | Female | 199 (46.3%) | 153 (35.6%) | 78 (18.1%) | 430 |
| | Male | 20 (21.5%) | 56 (60.2%) | 17 (18.23%) | 93 |
| Total | | 219 | 209 | 95 | 523 |

The above table demonstrates that very small number of patients showing the normal range (only those who used vitamin D supplements). The ratio of females having deficiency or insufficiency of vitamin D is more dreadful than males. The males are less affected than females at deficiency/insufficiency of vitamin D.

### 3.1.3 Other Findings

Through detailed medical history it was elucidated that all patients ascertained in present study came with the similar clinical symptoms like arthiritis, osteoporosis, diabetes mellitus, backache, backbone issues, joint pains and few with depression. It was analysed that most of the patients had swear joints pain and usage of vitamin D supplements helped to reduce the severity of pain. Indrop D injection (orally) were found to be most frequently used and effective supplement, recommended once or twice in a week depending on the situation of the patient. Moreover, it was observed that males and females responded equally well to vitamin D supplements.

## 3.2 Genetic Susceptibility/Etiology

### 3.2.1 Pedigree Analysis

The family ascertained here belonged to the Haripur but lived in Islamabad. The family had Awan baradari and had good socio-economic status. The reason behind to choose this family for the study was the presence of vitamin D deficient individuals in every generation. In addition to vitamin D deficiency, multiple disorders run in this family for example eye sight impairment, hearing impairment, cardio vascular problems, night blindness, breast cancer, foot drop, brain heamraghe and arthritis. Consanguineous marriages were not common in selected family. Total number of 41 blood samples were collected from this family in which four were vitamin D deficient and rest showed above mentioned clinical disorders. The affected individuals showed the symptoms of weak eye sight; swear joints pain. The pedigree of this family along with their haplotypes are shown in the figure 3.3.

The pedigree analysis showed that:

- Affected individuals had at least one affected parent.
- Dominant inheritance of affected individuals in all generation.
- It can affects either sex (male or female).

### 3.2.2 Linkage Analysis

The NOTCH 3 gene is found to link with dementia and because of the dementia association with vitamin D deficiency, markers D19S415 and D19S714 of this gene were run in the selected family to correlate the relation of the selected markers with vitamin D deficiency. The PCR product amplification of D19S415 marker is shown in figure 3.4.

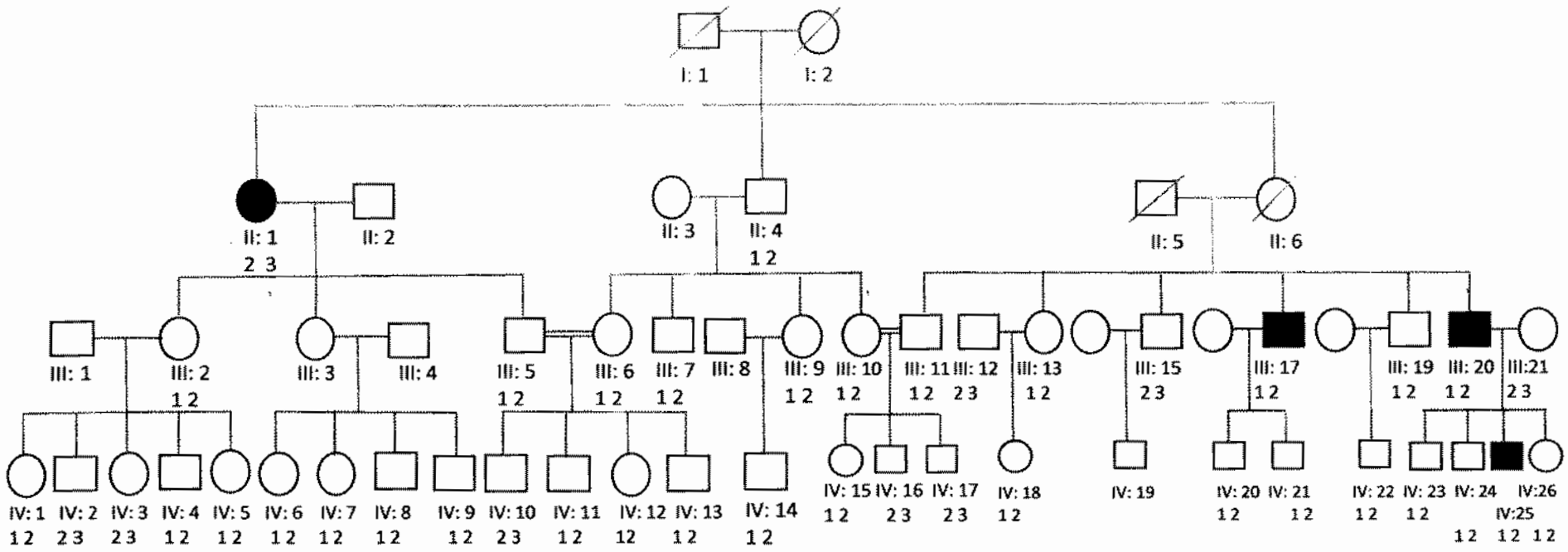


**Figure 3.3**: Haplotype Results of D19S415 marker in Vitamin D Deficient Family. Allele 2 is present in most of the individual with dominance inheritance pattern. D19S415 is not linked with this family. Square boxes represents the male individuals and circles represents the female individuals. Coloured boxes characterizes as the affected one while other are the normal. Similarly diagonal line over the boxes specify for the dead individual. Arabic Numbers showing the generation and along with the generation, individual numbers are present in numeric form. Below the individuals numeric numbers showing their haplotypes.


**Figure 3.4:** PCR amplification of vitamin D deficient family. 2% agarose gel result of D19S415 marker is shown in this figure with the amplification of 250bps fragment.

After the amplification, for linkage analysis the specific marker was screened in the family. The genotyping was accomplished with the help of PAGE (Poly Acrylamide Gel Electrophoresis) as mentioned in the chapter 2. The PAGE results of marker D19S415 is shown in the figure 3.5.

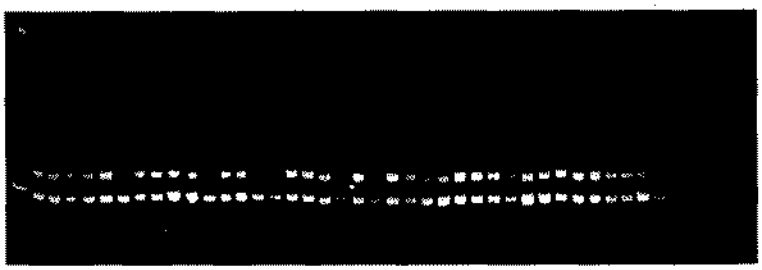

**Figure 3.5:** 12% PAGE results of maker D19S415. Specified marker run on affected and non-affected individuals for genotyping. Allele 2 is present in most of the patients as compared to allele 1 and 3.

### 3.2.2.1 Haplotype Analysis

When haplotypes at the result of both markers (D19S415 and D19S714) were analysed, it was observed that both markers were not linked with the selected family and allele 2 was present in most of the individuals with dominant inheritance pattern.

The presence of allele 2 in normal individuals and not causing vitamin D deficiency on them is indicating the non-linkage pattern. Furthermore, as night blindness, eye development impairment and cancer especially in females were found in this family, so it can be assume that these diseases were found owing to the presence of vitamin D deficiency in every generation of selected family. The haplotypes along with pedigree as a result of D19S714 marker is shown in figure 3.7.

### 3.2.3 Association Study

To check the genetic etiology for the deficiency levels of vitamin D, genetic association study was performed on random cases and controls samples. All samples were categorized in accordance to the specified range of vitamin D deficiency. SNP rs 10877012 of gene CYP27B1 (involved in the synthesis of $25(OH)_2$ D) was run on these random samples in order to find out the variation in genetic makeup of vit. D deficient patient (<12 ng/ml) then the patients having insufficiency range i.e. $\geq$ 12-30ng/ml. PCR amplification of these cases samples is shown in the figure 3.6. PCR products along with the specified primers were sent for sequencing at CAMB, Lahore but still sequencing results are being under process.


**Figure 3.6:** PCR amplification of vitamin D deficient cases samples. SNP rs 10877012 was run on these samples and 200bp fragment was amplified.

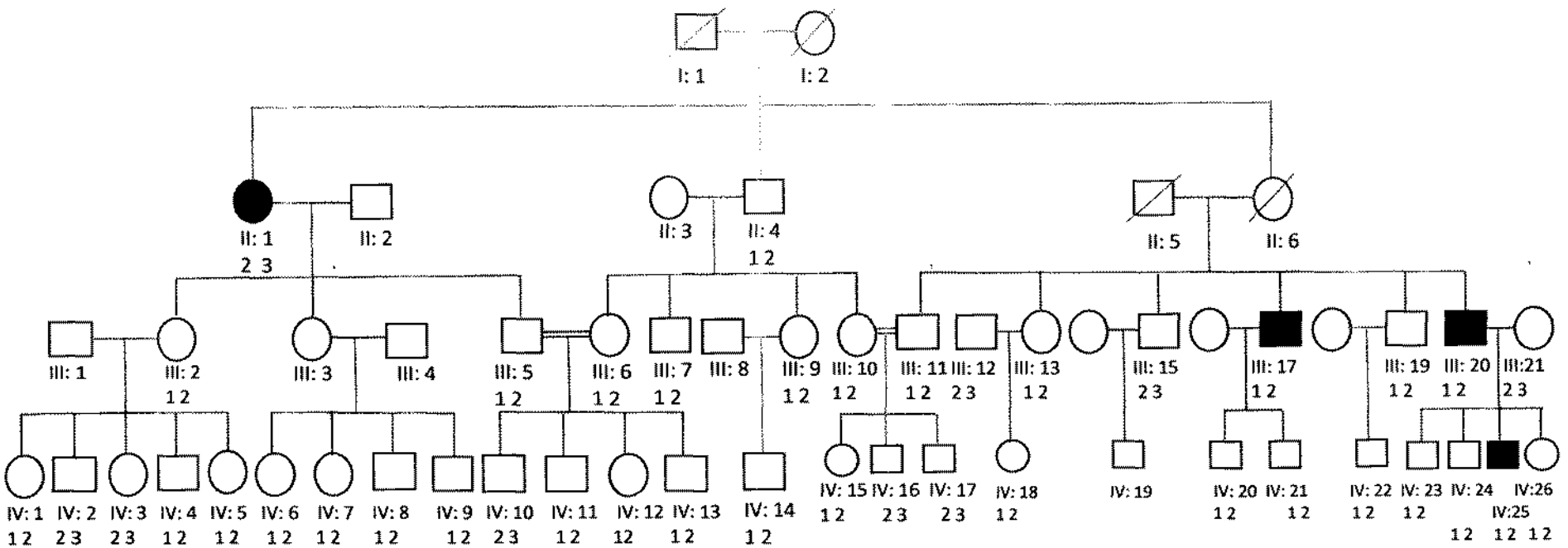


**Figure 3.7**: Haplotype Results of D19S714 marker in Vitamin D Deficient Family. Marker D19S714 is not linked in the selected family. Square boxes represents the male individuals and circles represents the female individuals. Coloured boxes characterizes as the affected one while other are the normal. Similarly diagonal line over the boxes specify for the dead individual. Arabic Numbers showing the generation and along with the generation, individual numbers are present in numeric form. Below the individuals numeric numbers showing their haplotypes.

# DISCUSSION

# 4 Discussion

Vitamin D deficiency is excessively gaining attention worldwide with the passage of time as it is known to be associated with osteoporosis, rheumatoid arthritis, diabetes mellitus (type I and type II), multiple sclerosis, weakness of muscles, depression, dementia and rickets in children. It is neither age specific nor gender specific. From the previous findings, it came to know that 1 billion people either had insufficient amount of vitamin D or they were vitamin D deficient (Holick, 2006). Despite the fact that Pakistan had immense amount of sunlight throughout the year, still there were vast number of vitamin D deficient cases. Vitamin D deficiency could be multifactorial in population i.e. genetic as well as environmental (Khan *et al.*, 2013). So keeping in mind the current situation of vitamin D deficient cases, the research was accompanied to review the etiology of deficiency of vitamin D in Pakistani population. NOTCH 3 gene played role in dementia and vit. D deficiency is considered one of the risk factor of dementia and different pshychratic problem. Therefore we also want to check the relation of NOTCH 3 gene with deficiency of vitamin D in vitamin D deficient family. The study comprises of two sections, firstly the linkage study on vitamin D deficient family, secondly, the association study on cases samples. The subjects included for association study were vitamin D deficient either they were diagnosed this deficiency recently or had the history of vitamin D deficiency. The included individuals were regardless of the age and gender limitations.

The linkage analysis of selected family revealed that two markers of NOTCH3 gene (D19S415 and D19S714) were not the causative agent for vitamin D deficiency as these markers did not show any linkage in this family. However it is reported that NOTCH3 gene is involve in vascular dementia and has been associated with vitamin D deficiency worldwide (McCann *et al.*, 2008) but the findings of current study didn't revealed NOTCH3 gene as a responsible element for vitamin D deficiency in selected family. The linkage study is done to

find out the ultimate consequences of diseases so that after family study we can figure out the chances of occurrence of specific disease and can help by genetic counselling and genetic screening enable to accomplish this counselling.

A research was accompanied in Australia in which 11,247 national individuals were enrolled and exhibited 31% prevalence of vitamin D deficiency. In addition, out 11,247 individuals 42% were females and 27% were males (Daly *et al.*, 2012). Another study conducted by Khan and his colleagues in 2013, whose results showed that ratio of the females (76.2%) were high than the males in Islamabad (23.8%) who had vitamin D deficiency (Khan *et al.*, 2013). These results are the evidence to support the findings of the present study i.e. females were more affected than the males with ratio of 82% and 18% respectfully.

In present study calculated mean for age of onset of the patients was 44 years (range 15- >75) that was prone towards deficiency of vitamin D. Previous findings showed that vitamin D deficiency was not age specific owing to the presence of different mean ages of patients. Although it had been noticed that levels of vitamin D significantly lower in the older patients as they had been associated with osteoporosis and bone related diseases (Baumgart *et al.*, 2011; Cantorna, 2012; Carvalho *et al.*, 2013). High ratio of vitamin D low level in old patients is also because skin morphology changes with the age and their skin absorbance is also reduces. The amount of precursor cholesterol molecule named 7-dehydrocholestrol also reduces as age increased (MacLaughlin and Holick, 1985). In one study of Mahmood and his colleague in Karachi, among 244 patients the mean age was 33.62 years (Mahmood *et al.*, 2009). Similarly, in another study of Islamabad, mean age was 36.3 years among 562 vitamin D deficient subjects (Khan *et al.*, 2013). According to an association study of vitamin D insufficiency/deficiency in France, mean age of onset was 46 lies in the range of 18-86 years (Foucan *et al.*, 2013). A cross sectional study was conducted in Danish families in which children had mean age of 10 years (range 4-17), among adults it was 41 years (range 18-60)

and collectively 30 years was the calculated mean age (Nissen *et al.*, 2013). The association study between polymorphism of CYP27B1 gene and HLA-B27-associated uveitis also showed that their subjects exhibited mean age of 44.8 years (Steinwender *et al.*, 2013).

The analysis of present study elucidated the fact that levels of vitamin D can be linked to the caste of an individual because Sheikh and Pathan caste were existed in greater amount as compared to rest of the caste. This can be owing to their genetic make-up which are not enough susceptible to absorb vitamin D deficiency in their skin. The relation of caste with vitamin D is analyse for the very first time as none of-the author focus on this variable.

The high prevalence of vitamin D deficiency/insufficiency in Pakistani population was observed in current data and this observation was matched with previous findings where low levels of vitamin D was demonstrated in local population as well as worldwide (as discussed in-detail in chapter 1). Similarly, increased amount of patients with poor sunlight exposure were noticed in present study which highlighting the fact that vitamin D deficiency mainly due to the inadequate sunlight exposure not due to less bioavailability. Other reasons behind this significantly low vitamin D levels can be demonstrated as dark skin tone need the prolonged sunlight exposure in order to reach the desired level of vitamin D. In addition, use of sun block, in door work and dressing style can also effect the vitamin D absorption because female wearing hijab can expose only hands or feet so they have reduce area towards sunlight. Besides these external features, any problem in vitamin D absorption metabolism or in any enzymatic activity (hydroxylation/dehydroxylation process in liver and kidney) can leads towards the low serum level of vitamin D (Iqbal & Khan, 2010).

Literature revealed the fact that Body Mass Index (BMI) is inversely proportional to vitamin D meaning thereby lower the serum vitamin D higher will be the BMI i.e. the subject will be towards obesity (Wortsman *et al.*, 2000). The results of current study were in contrast

to previous findings as patients were came with normal body mass index but they exhibited the low vitamin D level <12 ng/ml or =12-30ng/ml.

The relation of smokers with higher level of vitamin D was observed in one report. As smoking is prohibited in indoor public and work spaces so smokers use to do smoking in outdoor spaces, therefore, smoker's sunlight exposure is in great amount as compared to non-smokers. This aspect heading towards the high level of vitamin D among the smokers (Carvalho *et al.*, 2013). On the other hand there were also reports in which smoking had been associated to low levels of vitamin D (Janssens *et al.*, 2010; McCullough *et al.*, 2010). However, in current study there were very few patients who had smoking habit.

# 5 Conclusion

The prevalence of vitamin D deficiency is very high in Pakistan as well as Worldwide. Vitamin D deficiency is an alarming problem and its deficiency leads towards various serious disorders like osteoporosis, cancer, multiple secrosis, depression, type I diabetes, dementia, cardio-vascular diseases etc. Therefore, corrective actions are need to cope with this emerging deficiency. The major reason behind this high prevalence in our population is due to the genetic difference among different races, caste and ethnicities. People belonging to different races/ethnicity have genetic difference in absorption level of various nutrients including vitamin D deficiency. Besides these, the inadequate exposure of individuals towards sunlight also responsible for vitamin D deficiency as sunlight is considered to be its chief source. Results of current study reveal that genetic linkage of D19S415 and D19S714 markers of NOTCH3 gene is not found in selected family but overall allele 2 with dominant inheritance pattern is observe. Statistical analysis demonstrated that deficiency of vitamin D is not age specific but it can be easily notice that the older patients are more affected with it. Females are more prone towards this deficiency in every age group. The insufficient exposure towards sunlight is found in most of the patients. The number of patients having vitamin D deficiency showing highest percentage instead of vitamin D insufficiency. Vitamin D supplements is very helpful in maintaining vitamin D level in blood. The most common symptom that moves towards vitamin D deficiency is found to be joints pain.

# 6 Future Perspectives

Considering the significance of vit. D and the low susceptibility in our population, it is needed to increase its bioavailability and solubility in order to get its desire quantity. One of the strategy to increase its bioavailability is via nanotechnology as it is highly relevant that the solubility and bioavailability of various bio-actives could be increased by creating nano-delivery systems. Therefore, the future perspective of this project will be to design a delivery system for vitamin D with enhanced solubility for vitamin D as well as its bioavailability.

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