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Prevalence of Vitamin D Deficiency and Associated Risk Factors Among Patients with Nonspecific Musculoskeletal Pain Symptoms Attending Tikur Anbessa Specialized Hospital, Addis Ababa , Ethiopia

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This is to certify that the thesis prepared by Amanuel Dubale, entitled: *“Prevalence of Vitamin D deficiency and associated factors among patients with nonspecific musculoskeletal pain symptoms attending Tikur Anbesa Specialized Hospital in Addis Ababa; Ethiopia”* and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Clinical chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations and acronyms

1, 25 (OH)₂D: 1, 25-dihydroxy vitamin D

25OHD: 25-hydroxy vitamin D

ALP: Alkaline phosphatase

BMI: Body mass index

EPHI: Ethiopian public health institute

FGF-23: fibroblast growth factor 23

IGF1: insulin like growth factor 1

IGFBP3: insulin like growth factor binding protein 3

NCX1: sodium calcium ion exchanger 1

NPT2b: sodium phosphate transporter 2b

PMCA1b: plasma membrane calcium ATPase 1b

PTH: parathyroid hormone

RANK: receptor associated nuclear factor $\kappa\beta$

RANKL: receptor associated nuclear factor $\kappa\beta$ ligand

SOCC: source operated channel

SOP: standard operational procedure

TASH: Tikure Anbesa Specialized Hospital

TRPV6: transient receptor protein cat ion channel subfamily V member 6

UV: ultraviolet

VDBP: vitamin D binding protein

VDCC: voltage dependent calcium channel

VDR: vitamin D receptor

VDRE: vitamin D response elements

WC: waist circumference

Abstract

Background: Vitamin D deficiency causes rickets in children, and osteomalacia in children and adults. In 2016 musculoskeletal conditions are second cause of disability worldwide. Vitamin D deficiency and insufficiency can be both corrected and prevented safely through supplementation therapy. Therefore assessing prevalence of vitamin D deficiency and associated risk factors among patients with nonspecific musculoskeletal pain symptoms is crucial in Ethiopian context.

Objective: To determine the prevalence of vitamin D deficiency and its associated risk factors in patients with nonspecific musculoskeletal pain symptoms in Tikure Anbessa Specialized Hospital (TASH), Addis Ababa ; Ethiopia.

Methods: A cross-sectional study was conducted among patients with nonspecific musculoskeletal pain symptoms from March to May 2018 at TASH. Demographic data were collected using a structured questionnaire. Clinical data were obtained from medical records. Anthropometric indices were measured. Alkaline phosphatase (ALP) was determined in TASH laboratory with Mindray-200E. Vitamin D was determined with Mini Vidas hormone analyzer in Ras Desta Memorial hospital laboratory. Calcium (Ca) and Phosphorous (P) were analyzed in EPHI laboratory with Cobas 6000 c501 module. The data were entered into SPSS version 20 for analysis.

Results: Among the total 75 study participants with nonspecific musculoskeletal pain symptoms, 66 (88%) had vitamin D deficiency and 9 (12%) had vitamin D insufficient. Age, body mass index (BMI), waist circumference (WC) and occupation were associated with vitamin D deficiency. However, ALP, Ca, and P levels were not significantly correlated with vitamin D level. BMI were identified as independent risk factors for vitamin D deficiency.

Conclusion: The prevalence of vitamin D deficiency was high among patients with nonspecific musculoskeletal pain symptoms in this tertiary care. Obesity was independent risk factors for vitamin D deficiency.

Keywords: Nonspecific musculoskeletal pain symptoms, vitamin D deficiency, prevalence, risk factors

1. Introduction

1.1. Background

Vitamin D (D_2 and D_3) is cholesterol resembling molecule that physiologically regulates Calcium, Phosphorous, and bone metabolism. Vitamin D is gained from exposure of skin to sunlight, diet, and dietary supplements(1). Few foods naturally contain or are fortified with vitamin D meanwhile the main source is exposure to sun(2).

The ultraviolet (UVB) spectrum of sunlight (wave length 290-315 nm) radiates on human skin and facilitates the conversion of 7-dehydrocholesterol to pre-vitamin D, which undergo thermal isomerization to vitamin D in skin(3, 4). Vitamin D from dietary sources is incorporated into chylomicrons and transported by the lymphatic system into the venous circulation. With vitamin D made in the skin dietary vitamin D can be stored in and released from fat cells. Once vitamin D is ejected out of the plasma membrane in to the extracellular space, it bounds to the vitamin D binding protein (VDBP)(5). In the liver, vitamin D is converted by 25-hydroxylase (CYP2R1) to 25-hydroxyvitamin D (25OHD)(4). 25OHD is the major circulating form of vitamin D that is used to measure vitamin D status(6). This immediate precursor metabolite is hydroxylated to the active form of vitamin D [1, 25- dihydroxyvitamin D ($1, 25(OH)_2 D$)] by the mitochondrial 25OHD 1α -hydroxylase (CYP27B1) enzyme in the kidney(4).

25OHD 1α -hydroxylase is also expressed outside kidney in different tissues and enables the production of $1, 25(OH)_2 D$. This active form of vitamin D is locally active and exerts auto- or paracrine effect(7).

The renal synthesis of $1, 25(OH)_2 D$ is regulated by several factors including serum phosphorus, calcium, fibroblast growth factor 23 (FGF-23), parathyroid hormone (PTH) and vitamin D itself(2). $1,25(OH)_2 D$ induces its own destruction by rapidly inducing the 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), which leads to the multistep catabolism of both 25(OH)D and $1,25(OH)_2 D$ into biologically inactive, water-soluble metabolite(6).

Vitamin D exerts its genomic effect on target tissues that express VDR and non-genomic effects on tissues that express membrane bound vitamin D receptors. The target tissues include the classical tissues like intestine, bone, kidneys and Parathyroid gland and other non-classical tissues through the body that express its receptor including muscle and neurons (7).

Vitamin D prevents hypocalcaemia by increasing intestinal and renal calcium absorption and regulates the PTH levels. The role of vitamin D in metabolism of calcium and phosphorous leads to an adequate calcium-phosphorus product ($\text{Ca}^{2+} \times \text{HPO}_4^{2-}$) resulting in an effective bone mineralization hence keep bone integrity and structure(8). In muscles 1, 25(OH)₂D regulate gene transcription and rapid non-genomic pathways that affect the muscle cell regeneration and calcium handling(9).

Although there is no consensus on optimal levels of 25-hydroxyvitamin D as measured in serum, vitamin D deficiency is defined by most experts as a 25-OH D level of less than 20 ng/ml (50 nmol/L) and vitamin D insufficiency as 25-OHD of 20-29 ng/ml(50-75 nmol/L)(4). Symptoms of vitamin D deficiency can be nonspecific and include fatigue, altered mood and depression, insomnia, non-radicular back pain, arthralgia, proximal muscle weakness, headache, and hair loss(10).

Nonspecific musculoskeletal pain refers a pain for which no specific causes such as trauma, infection, tumor, and inflammatory disorders could be identified. The symptoms includes low back pain, knee pain and diffuse pain(11).

Vitamin D deficiency can lead to musculoskeletal pain due to bone demineralization and muscle weakness associated with hypocalcaemia. Direct effect of 1, 25(OH)₂D on muscle cells, neurons, neurotrophins, prostaglandin and nitric oxide is also another mechanism(8).

Different factors are associated with vitamin D deficiency which includes factors that decrease coetaneous synthesis of the vitamin, dietary intake and factors that decrease absorption of the vitamin(1).

1.2. Statement of the problem

The prevalence of Vitamin D deficiency in world general population is estimated to be between 25% and 50%(1). In developing countries, the prevalence of Vitamin D deficiency ranges between 30–90%. Despite ample sunshine throughout the year, one-third to one half of individuals living in Sub-Saharan Africa and the Middle East have serum 25-hydroxyvitamin D levels <25 nmol/l(12).

Even though Ethiopia is located to equator, Vitamin D insufficiency was reported to be 84.2% among women in southern Ethiopia(13). This result shows living near the Equator does not guarantee adequate vitamin D status.

Vitamin D deficiency causes rickets in children and osteomalacia in children and adults(2). The prevalence of rickets is high in many parts of Asia, Africa, and the Middle East and resurgence of rickets has been recorded even among the developed countries like Northern European countries. The global prevalence of osteomalacia in adults is assumed in regions where rickets is prevalent in children especially among pregnant women and the elderly(14).

A meta-analysis of observational study published in 2015 by Ming-Yen Hsiao and colleagues summarized that there was significantly higher risk of vitamin D deficiency in patients with chronic widespread pain than the control group(15). Another systematic review and meta-analysis of 29 studies published in the year 2017 by Joshua Zadro and colleagues summarized that there is strong association between vitamin D deficiency and low back pain(16).

In 2016 musculoskeletal disorders are second cause of disability worldwide with back pain as most frequent condition. The disability due to musculoskeletal condition is increased by 19% from 2006 to 2016 and is expected to rise in sedentary and aging people(17). One form of musculoskeletal disorder, low back pain's prevalence among Africans is rising and is of concern(18).

Musculoskeletal conditions produce severe long-term pain. It is well known that prolonged untreated or under-treated chronic pain can have significant negative physical impacts like long term activity limit, sleep disturbance and physical fatigue, psychological impacts like pain related anxiety and fear, and social effects like reduced time in leisure activities with family and the loved ones(19).

Vitamin D status has become an essential aspect of primary care. Vitamin D deficiency and insufficiency can be both corrected and prevented safely through supplementation(20). An endocrine society recommended screening for vitamin D deficiency in individuals at risk for deficiency and recommended dietary and supplement intakes of vitamin D for patients at risk for vitamin D

deficiency(21). In resource limited countries like Ethiopia it is difficult to screen for vitamin D deficiency for every individuals but screening for at list patients with symptoms of vitamin deficiency can identify patients with nonspecific musculoskeletal pain symptoms who can benefit from vitamin D supplementation.

Vitamin D deficiency and its associated risk factors among patients with nonspecific musculoskeletal pain symptoms are largely under studied in Africa despite the continent having a high burden of vitamin D deficiency (18) and Ethiopia is no exception. Therefore assessing prevalence of vitamin D deficiency and associated risk factors in nonspecific musculoskeletal pain symptoms is crucial in Ethiopian context.

1.3. Significance of the study

Prevalence of Vitamin D deficiency and its associated risk factor among patients with non-nonspecific musculoskeletal pain symptoms is under studied and to our knowledge there is no study done so far in Ethiopia. The data generated from this research showed the prevalence of vitamin D deficiency and its associated risk factors in patients with nonspecific musculoskeletal pain symptoms in TASH. Therefore, determining prevalence and risk factors of Vitamin D deficiency will alert clinicians and health policy makers to give attention to this problem and initiate the need for further research, appropriate intervention and its inclusion as part of routine diagnosis in patients with nonspecific musculoskeletal pain symptoms.

Vitamin D is measured rarely in Ethiopia in spite of suggestion by Endocrine society guideline to screen for vitamin D deficiency for individuals at risk of deficiency. Measuring vitamin D among patients with nonspecific musculoskeletal pain symptoms is important to identify patients who can benefit from vitamin D supplementation therapy.

Furthermore, the finding of this study will also provide background data for future research among this population with large sample size.

2. Literature review

2.1. Vitamin D and the Regulation of Calcium, Phosphorus, Bone and muscle function

Vitamin D increases the efficiency of intestinal calcium absorption from 10-15% to 30-40% and absorption of phosphorus from 60% to 80%(4).

In intestine calcium is absorbed through paracellular and transcellular transport pathway. In paracellular transport pathway Calcium enters enterocytes by passive diffusion while in the transcellular transport pathway calcium enters by active transport mediated by transporter proteins that are expressed due to stimulation by $1,25(\text{OH})_2\text{D}$ (22). The binding of $1,25(\text{OH})_2\text{D}$ to VDR modulates the transcription of genes encoding transporter proteins such as transient receptor protein cat ion channel subfamily V member 6 (TRPV6), calbindin, plasma membrane calcium ATPase 1b (PMCA1b) and sodium calcium ion exchanger 1 (NCX1)(23).

TRPV6 on the luminal surface of enterocytes facilitates the entry of calcium into the enterocytes and calbindin transports calcium from the luminal surface to the basal surface while PMCA1b and NCX1 extrudes the calcium from the basal surface to the extracellular circulation [Fig 1a](3).

Even though intestinal phosphate absorption is not tightly regulated as serum calcium, in severe hypophosphatemia, phosphorus is absorbed through active transport, which is facilitated by $1,25(\text{OH})_2\text{D}$. A large fraction of dietary phosphate intake is considered to be transported by a passive paracellular pathway. The transcellular pathway consists of sodium phosphate transporter 2b (NPT2b) [Fig 1b]. $1,25(\text{OH})_2\text{D}$ increases the expression of NPT2b transporter at the intestinal brush border membrane(22).

Vitamin D in kidney enhances the re-absorption of calcium by initiating expression of TRPV5 and calbindin transporter proteins(23). By negative feedback mechanism, vitamin D also regulates its production by inhibiting renal expression of 1α -hydroxylase(6).

PTH is one of the hormones involved in calcium homeostasis by inducing bone resorption and renal expression of 1α -hydroxylase. Vitamin D suppresses the proliferation of parathyroid gland cells and secretion of PTH by up regulating calcium receptors (CaR) on parathyroid gland cells. Calcium binds with CaR and suppresses the release of PTH(2).

Vitamin D maintains bone health by involving both in bone formation and resorption processes. In bone formation process vitamin D provides Ca and P to bone mineralization from intestine and kidney(24). The suppression of PTH by vitamin D, favors the bone formation process(3). Direct involvement of vitamin D in gene expression of bone forming proteins such as calcium binding

proteins and transcription factors for osteoblast differentiation is another mechanism of vitamin D involvement in bone formation(25). ALP is one of the bone differentiation transcription factors whose expression is stimulated by $1, 25(\text{OH})_2\text{D}$ (26) Though, measurement of increased ALP expression is taken as a reliable indication of the osteoblastic function the mechanism of this elevation in ALP expression is considered less clear. ALP was postulated to increase the local concentration of inorganic phosphate(27).

b)

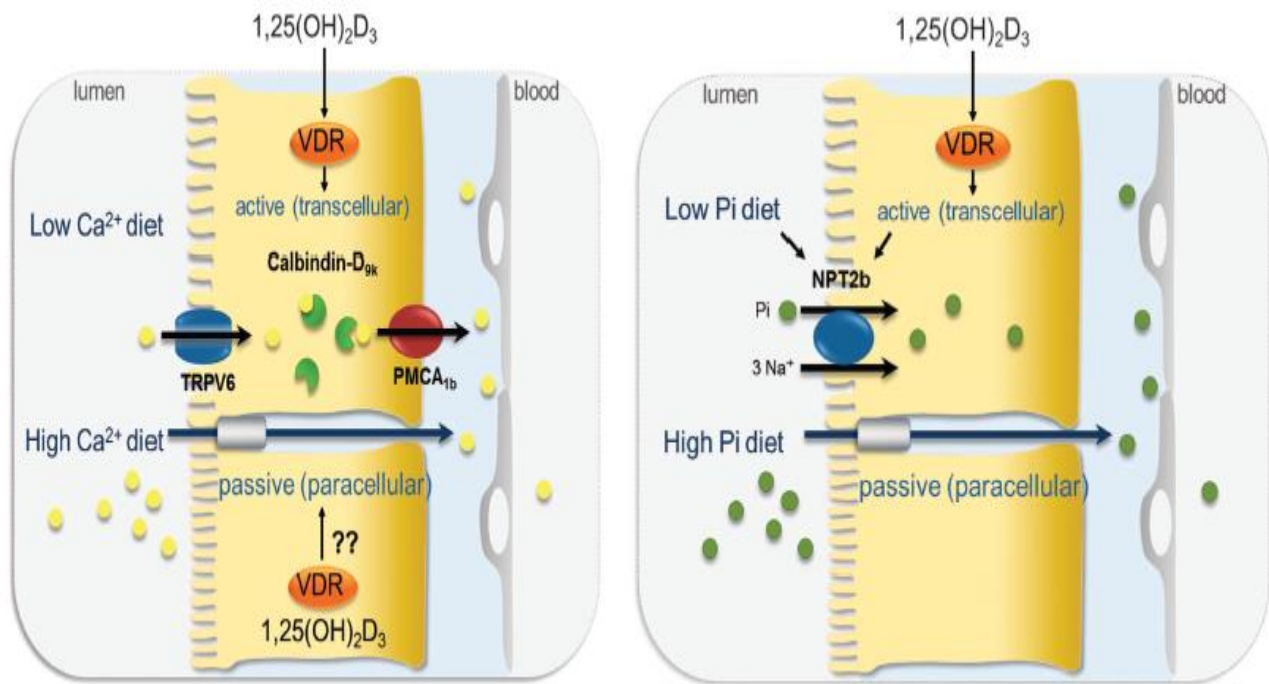


Figure 1: The role of vitamin D on intestinal absorption of a) calcium and b) phosphate (adapted from Christakos S, et al 2014)

To maintain serum calcium homeostasis vitamin D is also involved in mobilization of Ca and P from bone by stimulating osteoclastogenesis. Vitamin D up regulates expression of receptor associated nuclear $\kappa\beta$ transcription factor ligand (RANKL) in osteoblast progenitors and osteoblasts(3). By cell to cell contact between RANKL expressing osteoblasts and receptor associated nuclear $\kappa\beta$ transcription factor (RANK) expressing osteoclast progenitors, mature osteoclasts are differentiated. The mature osteoclasts dissolve the bone collagen matrix and release Ca and P to blood circulation. Vitamin D also down regulates osteoprotegerin which is the hormone that antagonizes RANKL(25).

Muscle cells are one of the cells known to express VDR(9). The binding of $1, 25(\text{OH})_2\text{D}$ to the VDR results in enhanced transcription of a range of proteins(26), including proteins involved in calcium metabolism, insulin like growth factor 1(IGF 1) and insulin like growth factor binding protein 3 (IGFBP 3). IGF 1 induces proliferation, differentiation and hypertrophy of skeletal muscle and is a

key component in muscle regeneration. IGFBP 3 binds IGF-1 in the serum. The formation of IGF-1–IGFBP-3 complex may block the binding of IGF-1 to its receptors, thereby mitigating its effect on DNA synthesis, growth and glucose regulation, but also prevent its rapid clearance(7).

Calcium is necessary for normal muscle contraction and relaxation. Vitamin D enhances muscle cell intracellular calcium homeostasis by indirect serum calcium homeostasis and direct non genomic calcium influx. The non-genomic calcium influx to muscle cell cytosol relies on vitamin D dependent activation of store operated calcium channel (SOCC) and voltage dependent calcium channel (VDCC) component of signal transduction. The SOCC component of signal transduction results in inositol phosphate 3 (IP3) –dependent calcium release from sarcoplasmic reticulum while VDCC results in phosphokinase A (PKA) dependent extracellular calcium influx(28).

2.2. Association of vitamin D deficiency with nonspecific musculoskeletal pain symptoms

Vitamin D deficiency results in abnormalities in calcium, phosphorus, and bone metabolism. Specifically, vitamin D deficiency causes a decrease in the efficiency of intestinal absorption of dietary calcium and phosphorus, resulting in an increase in PTH levels. PTH maintains serum calcium in the normal range at the expense of mobilizing calcium from the skeleton and increasing phosphorus wasting in the kidneys(19).

Vitamin D deficiency in children causes growth retardation and rickets. In adults, vitamin D deficiency causes a skeletal mineralization defect. The bone tissue with mineralization defect provides little structural support for the periosteal covering. As a result osteopenia, osteoporosis, and increase in the risk of fracture and osteomalacia; often complain of isolated or global bone discomfort along with aches and pains in their joints and muscles(23).

Deficiency of 1,25 (OH)₂D that maintains calcium homeostasis, modulates neuronal excitability, up regulates the synthesis of neurotrophins, influences prostaglandin action by inhibiting COX-2 expression and stimulates 15-prostaglandin dehydrogenase (15-PGDH) expression, and inhibiting synthesis of nitric oxide synthetase is another mechanism of nonspecific musculoskeletal pain symptoms(8).

A meta-analysis of observational study published in 2015 by Ming-Yen Hsiao and colleagues which included twelve studies comprising 1,854 patients with chronic widespread pain summarized that there was significantly higher risk of vitamin D deficiency than the control group(15). Another systematic review and meta-analysis of 29 studies published in the year 2017 by Joshua Zadro and colleagues summarized that there is strong association between vitamin D deficiency and low back pain(16).

A 2010 descriptive, analytical, cross-sectional study in Sao Paulo by Santos F C and colleagues found a high prevalence of vitamin D deficiency and insufficiency of 49 and 38%, respectively; however such levels were not significantly correlated to chronic pain(29).

Paul S. McCabe et al. recruited 3369 men aged 40–79 between 2003-2005 from eight European centers for a longitudinal study of male ageing and after adjustment for age and centers, physical performance and number of co morbidities, compared to those in upper quintile of 25-(OH) D (≥ 36.3 ng/mL), those in the lowest quintile (< 15.6 ng/mL) were more likely to develop chronic wide spread pain(30).

Demiryurek BE and Gundogdu AA. in their case control study included 36 patients with mild carpal tunnel syndrome and 40 without carpal tunnel syndrome in 2016 found significantly lower vitamin D levels in the mild carpal tunnel syndrome patients than those electro-physiologically normal subjects(31).

Sarah E. Tague et al conducted experimental study on rats. The rats were offered vitamin D-deficient diets for 2– 4 weeks and the rats showed mechanical deep muscle hypersensitivity but not coetaneous hypersensitivity. This result indicated that vitamin D deficiency can lead to selective alterations in target innervations, resulting in presumptive nociceptor hyper-innervations of skeletal muscle, which in turn is likely to contribute to muscular hypersensitivity and pain(32).

Noha T. Abokrysha studied 30 women diagnosed with fibromyalgia in Saudi Arabia in 2011. The result showed mean vitamin D level of 4.76 ± 1.46 ng/mL and significant negative correlation between vitamin D level and widespread pain index(33). Another hospital based case control study conducted in 2010 in Saudi Arabia by Ahmed et al showed that a mean serum ALP, PTH and serum 25 (OH) vitamin D levels were significantly difference in patients than controls(34).

A prospective, observational, triple-arm case-control study which included a total of 250 chronic low back pain, 177 sub-acute low back pain cases, and 248 controls conducted in south India by Ajay Panwar et al from November 2016 to January 2017 showed no significant difference in the prevalence of vitamin D deficiency among chronic low back pain, sub-acute low back pain, and controls which was 53.6, 50.8, and 51.6% respectively, in the three arms. However, the categorical analysis revealed that chronic low back pain and sub-acute low back pain cases had a significantly higher prevalence of worse categories of vitamin D deficiency(35).

A study done in south Australia by Jill Benson and colleagues in 2005 on eight cases with muscle pain and eight age and sex matched controls showed that all the eight patients with muscle pain had lower vitamin D levels than those without muscle pain(36).

2.3. Prevalence of vitamin D deficiency among patients with nonspecific musculoskeletal pain symptoms

A retrospective study done by Turner et al involved 267 of 313 patients admitted to the Mayo Comprehensive Pain Rehabilitation Center in USA for whom Vitamin D levels were available, revealed the prevalence of vitamin D deficiency as 26%(37). Another cross sectional study in USA by Gregory A. et al reported prevalence of vitamin D as 93 % among patients with persistent, nonspecific musculoskeletal pain(38).

Gorter EA et al conducted a cross sectional study in Netherland from 2012 to 2013 which showed out of 208 men and 319 females with fracture, 166 patients (31 %) had insufficient levels (50–75 nmol/l) and 210 patients (40 %) had a vitamin D deficiency (calcidiol <50 nmol/L), of whom 58 patients (11 % of the total group) had a severe vitamin D deficiency(39). A cross sectional study on 174 Switzerland chronic patients by Von Känel P et al reported 92% of the participants had vitamin D level below 30ng/ml from which 71% were vitamin D deficient and 21% vitamin insufficient(40).

Of 218 premenopausal women with common musculoskeletal pain and unknown etiology studied by Halim Yilmaz and colleagues in Turkey, 79.8% were detected with vitamin D deficiency(41). Another cross-sectional study by Muharrem Çidem and colleagues involved 8457 patients with widespread musculoskeletal pain showed the prevalence of vitamin D deficiency of 73.9%(42). A retrospective study on 145 Turkey chronic low back pain by Yalkın Çalık and Ümit Aygün reported 22.8% prevalence of vitamin deficiency and 22.8% insufficiency(43).

Among sixty Saudi males with musculoskeletal pain complains studied by Hala Lotfy Fayed and Amel Hamdy Saleh, fifty-four (90%) had vitamin D inadequacy; 42 (70%) deficiency and 12 (20%) had insufficiency and 6 (10%) had adequate 25(OH)D(44). Another similar study by Sadat-Ali M and colleagues on 187 Saudi females with nonspecific musculoskeletal pain showed 27.27% vitamin D insufficiency and 49.19% deficiency(45).

A descriptive analytic study performed on Sixty five adult patients with musculoskeletal pain symptoms in Qazvin city, Iran by Mahnaz Abbasi and his colleagues showed vitamin D deficiency of 95.4 %(46). Another cross sectional study by Alireza Jalili and colleagues in Iran on 438 patients nonspecific musculoskeletal pain showed 86% of the patients had vitamin D less than 30ng/ml and deficiency of 47%(11). A cross sectional study performed on 355 Iraq patients with chronic patients nonspecific musculoskeletal pain reported vitamin D prevalence of 85.35%(47).

Among forty female patients with fibromyalgia included in the study by Shaheen Ayuob Bhatti et al. in Karachi, Pakistan thirty two (80%) patients had Vitamin D deficiency, with mean levels of 15.855 ± 4.918 ng/ml and 8(20%) had Vitamin D insufficiency, with mean levels of 23.64 ± 2.39 ng/ml(48). Another study conducted by Sobia Qamar and colleagues in Karachi, Pakistan from October 2008 to September 2009 included hundred children aged 5-12 years, presenting with limb pains and fulfilling the diagnostic criteria of growing pains showed the prevalence of vitamin D deficiency of 72% and insufficiency of 22%(49). A case control study which involved 60 cases with persistent non-specific musculoskeletal pains and 60 controls reported 86.6% prevalence of vitamin D deficiency among the cases(50).

A cross sectional by Afsar SS. and colleagues in Pakistan on 500 patients with low backache showed 88.4% had vitamin D level below 30ng/ml and deficiency of 63%(51). Another cross sectional study in Pakistan by Humaira Achakzai and colleagues which involved 400 participants, reported 80% prevalence of vitamin D deficiency(52).

A study by Babita Ghai et al. in India was included 328 patients in an open label, single arm clinical trial aimed to assess the effectiveness of vitamin D supplementation in patients with chronic low back pain and determined prevalence of vitamin D as 86%(53).

Another cross sectional study was conducted among 281 adult patients above the age 20 years with non-specific complaints of general body pain/back pain/tiredness/weakness on working and walking with no other symptoms and no relief of the pain symptoms with routine treatments with analgesics and rest and physiotherapy by A Manoharan and colleagues in India. The result showed that out of 281 patients tested, 201 (71.5%) of the study population had below normal vitamin D of them 40.2% had deficiency, 31.3% had insufficiency and 28.5 had sufficient vitamin D level(24).

A cross sectional study conducted on North Korean refugees in South Korea reported all 386 participants had vitamin D level below normal (30ng/ml) of which 87% had vitamin D deficiency(54).

2.4. Risk factors associated with vitamin D deficiency

Factors associate with vitamin D deficiency includes, deprivation of sunlight, decline in coetaneous synthesis of vitamin D due to aging, and decrease in renal hydroxylation, melanin pigmentation, the solar zenith angle which depends on latitude, season, time of the day, low intake of foods fortified with vitamin D, obesity, smoking, pollution, exercise, use of drugs (e.g. anti-epileptic drugs and anti-tuberculous drugs) or diseases that accelerate vitamin D metabolism (e.g. reticulo-endothelial

malignancies, sarcoidosis, tuberculosis), renal insufficiency, mal-absorption syndromes, and chronic liver disease(1).

A review by A. Mithal et al summarized as older age; female sex; darker skin pigmentation; factors that determine sunlight exposure, such as clothing and cultural practices; dietary habits; and national policies of vitamin D fortification are risk factors for vitamin D deficiency(55).

BMI is associated with low vitamin D status. A cross-sectional study on US 18-year-old children by Christy B. Turer and colleagues showed Vitamin D deficiency is highly prevalent in overweight and obese children and severely obese (56). In another study in Singapore by Xinyan B and colleagues, BMI was significantly differ between those who were vitamin D deficient and those who were without Vitamin D deficiency ($P=0.047$) (57). Another study by Merlo C. and colleagues showed negative correlation between vitamin D and BMI ($r= -0.156$, 95%CI= -0.202 to -0.108)(58). On other side Keyeong Kim et al found Underweight participants (body mass index (BMI) <18 kg/m²) had significantly lower 25(OH) D levels than individuals with normal BMI($P=0.04$) (54).

In a survey conducted by the National Center for Health Statistics in US population, Vitamin D deficiency was significantly more common in not college educated($p<0.0001$) and obese ($p<0.0001$)(59). In another study by Catherine M. Gordon et al in USA on 307 healthy adolescents serum 25OHD levels were inversely correlated and body mass index ($r=-0.29$) (60).

A hospital based case-control conducted in Saudi Arabia from September 2010 to June 2011 by Ahmed M. S. Hegazy et al showed mean serum ALP and serum 25 (OH) vitamin D levels were significantly difference in patients with low back pain than controls without low back pain ($p<0.001$)(34).

In Qatar healthy school children studied by Abdulbari Bener and colleagues there was a significant difference between groups with deficient vitamin D and those with sufficient vitamin D in terms of physical activity, exposure to sunlight and duration of time spent outside under the sun($P<0.05$). The mean values of vitamin D serum concentration, calcium, alkaline phosphates, and phosphorus were very low in vitamin D-deficient children(61). On another hand a study by Sima Hashemipour et al in Tehran showed Vitamin D serum levels had no significant statistical relation with the duration of exposure to sunlight, kind of clothing and BMI(62).

A cross sectional study conducted by Silva Hovsepian and colleagues in Isfahan city, Iran showed Vitamin D deficiency was more prevalent among women ($p=0.001$) and younger age-group

($p=0.001$)(63). Another study by McCarty D. E and colleagues found age as independent risk factor for vitamin D deficiency(64).

Sobia Qamar and colleagues conducted a study in Karachi, Pakistan which showed that hypocalcaemia was found to be more common in patients with vitamin D deficiency but this was not found to be statistically significant ($p=0.863$) and ALP was elevated in 38 patients (38%)(49).

A study by Kapil U et al in India also showed significantly high vitamin D deficiency in females($p<0.001$)(65). On the other hand a study done in India by Babita Ghai and colleagues found that men were significantly more prone to have deficiency as compared to women(OR = 1.78 (1.10 – 2.88), $P = 0.02$)(53).

Tae-Hwan Kim et al identified a significantly higher prevalence of vitamin D deficiency in medical co- morbidity, and lower score for sunlight exposure($p<0.005$)(66).

Fadheela T Al-Mahroos and colleagues conducted study in Bahrain which showed significant association of high income (P-value 0.020), smoking (P-value 0.021), lack of sun exposure (P-value 0.001) and high body mass index (P-value 0.022) with vitamin D deficiency(67).

A retrospective study in Turkey by Atalay G S and colleagues showed that there was significant mean vitamin D difference between females and males($p=0.004$). However, the study reported no significant association between serum vitamin D level and Ca, P and ALP(68). Another study in Denmark by Brot C. et al also reported no relation between serum vitamin D metabolites, Ca and P (69).

A study in India by Lodh M and colleagues showed positive correlation between Vitamin D and Ca, P and ALP (70). Another cross sectional study in Qatar by Bener A and colleagues also reported significantly low mean vitamin D, Ca, P and ALP in vitamin D deficient group (61)

2.5. Conceptual framework

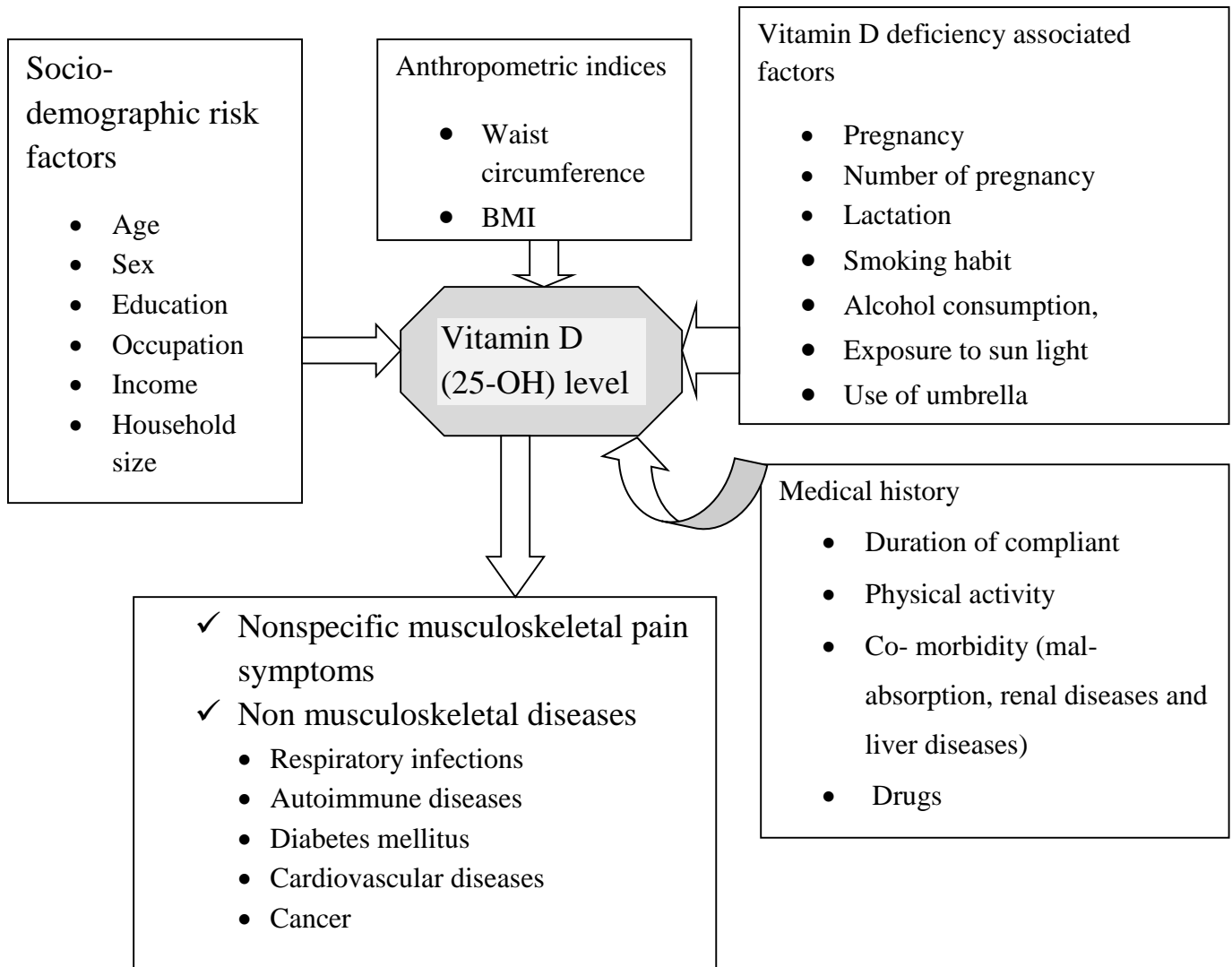


Figure 2: Conceptual framework

3. Objectives of the study

3.1. General objective

To determine the prevalence of vitamin D deficiency and its associated risk factors in patients with nonspecific musculoskeletal pain symptoms in TASH in Addis Ababa, Ethiopia from March to May 2018.

3.2. Specific objectives

- To determine the prevalence of vitamin D deficiency among patients with nonspecific musculoskeletal pain symptoms
- To compare the level of biochemical parameters among patients with nonspecific musculoskeletal symptoms
- To determine potential risks factors associated to vitamin D deficiency among patients with nonspecific musculoskeletal symptoms

4. Material and methods

4.1. Study design and period

A Cross sectional study design was conducted from March to May 2018.

4.2. Study area

This study was conducted in Tikur Anbessa Specialized Hospital. TASH is located in Addis Ababa. Addis Ababa is the capital and largest city of Ethiopia. TASH is referral and teaching hospital that serves approximately 370,000- 400,000 patients a year. The hospital has 800 beds, with 169 specialists, 65 non-teaching doctors. The hospital provides service with different clinics including neurology and rheumatology clinic(71).

4.3. Population

4.3.1. Source population

All patients who were visited TASH neurology and rheumatology clinic

4.3.2. Study population

All patients who were visited TASH neurology and rheumatology clinic for nonspecific musculoskeletal pain symptoms during the study period and met the inclusion criteria.

4.4. Exclusion and inclusion criteria

4.4.1. Inclusion criteria

- Patients who were with nonspecific musculoskeletal pain symptoms in TASH neurology
- Patients who were rheumatology clinic and capable of independent communication to give informed consent.

4.4.2. Exclusion criteria

- Patients with age of less than 18 years old
- Patients on vitamin D supplementation therapy
- Patients on calcium supplementation therapy
- Patients on multivitamin therapy

- Patients with known history of chronic diseases like liver failure and/or kidney failure
- Patients with rheumatologic diseases, trauma and anatomic skeletal malformations

4.5. Study variables

4.5.1. Dependent variables

Vitamin D deficiency

4.5.2. Independent variables

- Socio-demographic factors; age, sex, education, occupation, household size and income level
- Vitamin D deficiency associated factors; smoking habit, alcohol consumption, exposure to sun, dietary intake of fish and/egg, use of sunscreen and menopause state.
- Anthropometric indices; waist circumference and body mass index (WC, BMI)
- Medical history; duration of complaint, physical activity and co- morbidity

4.6. Measurement and data collection procedure

4.6.1. Sample size determination

The actual sample size for the study was determined using the formula for single population proportion by assuming 5% marginal error (d), 95% confidence interval ($\alpha=0.05$). The value of p is 95.4% from previous study conducted in Iranian patients with musculoskeletal pain symptoms(46). Based on the above information the total initial sample size is calculated by using the following formula.

$$N_i = \frac{[(Z \alpha/2)^2 pq]}{d^2}$$

Where; N_i = required initial sample size

$Z\alpha/2$ = critical value for normal distribution at 95% confidence interval which equals to 1.96 (Z value at $\alpha=0.05$).

p = the prevalence of vitamin D insufficiency in Iranian nonspecific musculoskeletal symptom patients= 95.4%

q = Proportion of Iranian nonspecific musculoskeletal symptom patients without deficiency of vitamin D= 0.46%

$d = \text{marginal error} = 0.05$

$$N_i = (1.96)^2 (0.46 \times 0.954) / [(0.05)^2] = 68$$

Taking 10% non-response rate, a total sample size is calculated to be 75.

4.6.2. Sampling method

Consecutive convenient sampling method was used to select 75 study participants.

4.6.3. Demographic and clinical data collection procedure

The demographic characteristics and associated risk factors data such as; age, gender, smoking habit, alcohol consumption, exposure to sun, education, occupation, level of income, household size, dietary intake of fish and/egg, were collected using a structured questionnaire by trained data collectors (**Refer Annex II**).

Duration of compliant, physical activity, and other co- morbidity were collected from the medical records by using checklist forms (**Refer Annex III section A**).

Anthropometric characteristics of study participants: height and weight were measured. BMI was calculated as weight (kg) divided by height squared (m²). WC was measured at a point midway between the inferior border of the costal margin and the iliac crest in mid auxiliary line to the nearest 0.5 cm. Collection of these medical records and clinical measurements were done by professional nurse to minimize bias. BMI of less than 18.5kg/m² was considered underweight, BMI between 25 and 29.9 kg /m² was considered overweight, whereas an adult who has a BMI of 30 kg /m² or higher was considered obese. Central obesity was assessed by WC and subjects with WC ≥ 94 cm was regarded as obese, according to the international guideline(72).

The questioner was translated into Amharic to make it easily to understand.

Vitamin D deficiency was defined as a 25-hydroxyvitamin D level of less than 20 ng per milliliter and vitamin D insufficiency as 25(OH) D of 20-29 ng/ml(4).

4.6.4. Blood sample collection, processing and laboratory analysis

Whole blood sample was collected from study participants using serum separator tube. After collection of 4 ml of blood sample, the sample was allowed to clot for 30 minute and serum was separated by centrifugation at 1500 rpm for 10 minutes. Then serum samples were stored in nunc tubes for biochemical test and immunoassay at -20 °C until analysis (**Refer Annex IV section A**). The activity of alkaline phosphatase was measured at TASH laboratory. Then the stored sample was transported to

Ras Desta Memorial Hospital Laboratory for analysis of Vitamin D and to Ethiopian public Health Institute clinical chemistry referral laboratory for analysis of calcium and phosphate.

Vitamin D (25- OH) was analyzed with mini Vidas hormone analyzer, by combined competitive enzyme immunoassay method with final fluorescent detection. To measure Vitamin D the solid phase receptacle serves as solid phase as well as pipetting device. The sample was mixed with pretreatment reagent to separate vitamin D from its binding protein. The pretreated sample is then collected and transferred to anti-vitamin D-ALP conjugate coated well. Then vitamin D in the sample was allowed to compete with the vitamin D in the solid phase for anti-vitamin D-ALP conjugate. During the detection the conjugate enzyme catalyzes the substrate in to fluorescent product which was measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of vitamin D in the sample. (**Refer Annex IV-section B**).

Alkaline phosphatase (ALP) was analyzed in TASH clinical chemistry laboratory with mindery by spectrophotometric method in which ρ -Nitrophenyl phosphate is hydrolyzed to p-nitrophenol and inorganic phosphate. The rate at which the ρ -Nitrophenyl phosphate is hydrolyzed measured at 405nm, was directly proportional to the alkaline phosphatase activity. (**Refer Annex IV-section C**)

Calcium (Ca) and Phosphate (P) were analyzed in EPHI national clinical chemistry laboratory with Cobas 6000 c501 module. Photometric method was used to analyze the analytes in the serum samples. Calcium ion reacts with 5-nitro-5-methyl-1, 2-bis (o-aminophenoxy) ethane-N, N, N, N-tetraacetic acid under alkaline condition to form a complex and this complex reacts with ethylendiaminetetraacetic acid. Change in absorbance is measured photometrically at 340 nm (**Refer Annex IV section D**). P reacts with ammonium molybdate complex in the presence of sulfuric acid and the amount of phosphomolybdate formed was measured photometrically at 340 nm which was directly proportional to the P in sample (**Refer Annex IV section E**)

4.7. Data quality assurance

The questioner was translated into Amharic to make it easily to understand when administered to study participants being assisted by data collectors. The data collectors were professional nurses who work in neurology clinic. The data collection instruments were pre-tested for coherence and data collectors were trained and supervised by principal investigator during the data collection period. The data collected by questionnaire were checked for completeness.

Pre-analytical: Blood sample quality was ensured during collection and processing by following standard operating procedures. Samples were stored in appropriate refrigerator temperature at -20°C until analysis in Ras Desta Memorial Hospital and EPHI laboratories.

An ice box was used for transportation of the sample to Ras Desta Memorial Hospital and EPHI

Analytical: the automated clinical chemistry analyzers (mini Vidas hormone analyzer, mindery and cobas) were calibrated and their performances were checked by running quality controls.

Post analytical: results were printed out after checking appropriateness of all the test results and the data were carefully entered into Microsoft Excel worksheet and saved for statistical analysis. The leftover samples were discarded according to the protocol of waste disposal of the respective laboratories.

4.8. Data processing, presentation and analysis

Data which were obtained from medical records, using questioners and findings from laboratory analysis were checked for completeness and consistency, coded and entered into Microsoft Excel and were exported to SPSS for analysis.

Before data analysis was carried out, all continuous variables were assessed whether they are normality distributed or not visually using histogram. Data were also explored and tested by Kolmogorov-Smirnov (K-S) and Shapiro-Wilk test for normality. If $p \geq 0.05$, the data were considered as normally distributed.

Descriptive statistics were used to summarize Qualitative variables and were reported with number and percentages. Graphs and tables were used to present results. All parametric values were expressed as the mean \pm SD whereas for non-parametric values median were reported with interquartile range. Fisher's exact (Ψ) test was also used to compare qualitative variables. Pearson's and Spearman's correlation for parametric and nonparametric data were used to establish correlations respectively. Independent sample t-test was used to compare parametric data. Multiple logistic regressions were used to identify independent risk factors for vitamin D deficiency. P value < 0.05 was considered statistically significant.

4.9. Ethical considerations

Ethical clearance was obtained from the Departmental Research and Ethics Review Committee of the Department of Medical Laboratory Sciences, School of Nursing and Midwifery of Addis Ababa University. This ethical clearance and support letter was submitted to the Department of Neurology of

Addis Ababa University College of health sciences; the department was further evaluated the proposal of this research and allowed the start of the research by a letter which was written to TASH neurology and rheumatology clinic.

Written consent was obtained from each study subjects before collection of blood samples and other relevant clinical information. Study participants were recruited only based on their consent and the inclusion and exclusion criteria apart from any form of discrimination. Security and confidentiality of detail clinical and laboratory finding data was kept carefully by using code for identification.

The participants were ensured to be free from any coercion, under inducement, influence or intimidation. The only potential risk was a little pain due to vein puncture and consumption of time of participant during the data collection.

The participants had the right to withdraw from the study whenever they feel inconveniences.

The results of participants with vitamin D deficiency were linked to the attending physician for appropriate treatment and management.

The leftover sample of the participants were decontaminated and disposed according to waste disposal protocol of respective laboratories immediately after investigation of the research parameters.

4.10. Result dissemination

The result of this study will be presented for thesis defense, conferences, stakeholders, and publication on peer reviewed journals.

4.11. Operational Definitions

Nonspecific Musculoskeletal pain symptoms: nonspecific pain for which no specific causes such as trauma, infection, tumor, and inflammatory disorders could be identified.

Central obesity: subjects with WC \geq 94 cm

Obese: who has a BMI of 30 kg/m² or higher.

Overweight: BMI between 25 and 29.9 kg/m²

Underweight: BMI of less than 18.5kg/m²

Vitamin D deficiency: a 25-hydroxyvitamin D level of less than 20 ng per milliliter (50 nmol per liter)

Vitamin D insufficiency: a 25(OH) D level of 20-29 ng/ml

Vitamin D sufficiency: a 25(OH) D level of >30 ng/ml

5. Results of the study

5.1. Socio-demographic and anthropometric characteristics of study participants

The minimum and the maximum age of study participants were 18 and 80 year respectively. The mean age was 48.89 years (Table 1).

Table 1: Socio-demographic and anthropometric characteristics of patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May 2018 (n=75)

No.	Variables	Categories	Frequency	Percent
1.	Age (Years)	18-35	13	17.3
		36-55	39	52
		≥55	23	30.7
2.	Sex	Male	24	32
		Female	51	68.0
3.	Menopause stage	No	20	26.7
		Yes	31	41.3
4.	Level of Education	Illiterate	10	13.3
		primary school	28	37.3
		Secondary school	18	24.0
		College/University	19	25.3
5.	Occupation	Government employee	22	29.3
		Private business	17	22.7
		Unemployed	6	8.0
		Housewife	19	25.3
		Farmer	2	2.7
		Retired	9	12
6.	Monthly income(ETB)	<2100	50	66.7
		≥2100	25	33.3
7.	Household size	≤5	48	64.0
		>5	27	36.0
8.	BMI (Kg/m2)	<18.5	35	46.7
		25-29	21	28.9
		>29	14	18.7
9.	WC	<94	48	64
		≥94	27	36

BMI: body mass index, WC: waist circumference

5.2. Life style and clinical characteristics of study participants

Of 75 study participants 54(72%) had sun exposure of less than or equal to 2.4 hours per day. The majority of study participants 72 (96%) didn't use sun screen. Of the participants 62(92%) were never smoked and 25(33.3%) were drinking alcohol currently. However, 46(61.3%) were reported never drunk. Majority of the study participants 50(66.7%) had less than three years of duration of pain complaint (**Table 2**).

Table 2: Life style and clinical characteristics of patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May 2018. (n=75)

No.	Variables	Categories	Frequency	Percent
1.	Time of sun exposure(hours)	≤2.4	54	72
		>2.4	21	28
2.	Use of Sun screen	Yes	3	4
		No	72	96
3.	Fish intake per month	Never	53	70.7
		<1	2	22.7
		1	4	5.3
		>1	1	1.3
4.	Egg intake per week	Never	31	41.3
		<1	23	30.7
		1	12	16
		>1	9	12
5.	Habit of cigarette smoking	Current	1	1.3
		Past	5	6.7
		Never	69	92
6.	Habit of alcohol consumption	Current	25	33.3
		Past	4	5.3
		Never	46	61.3
7.	Duration of the pain	<3	50	66.7
		3-6	19	25.3
		>6	6	8
8.	Other co-morbidity	No	42	56
		HIV AIDS	4	5.3
		Hypertension	15	20
		Diabetes Mellitus	10	13.3
		Others*	4	5.3

*breast cancer, coronary artery disease and asthma

5.3. Biochemical characteristics of study participants

The mean calcium and phosphorous was 2.08 ± 0.021 and 1.13 ± 0.192 respectively whereas the mean Vitamin D was 15.051 ± 4.67 . The median ALP activity was 204 with inter quartile range of 161-288. (Table 3).

Table 3: Biochemical characteristics of patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May, 2018 (n=75)

No.	Variables	Categories	Frequency	Percent	Mean \pm SD	Range	Median (IQR)
1.	ALP (IU/L)	<23	1	1.3		1142	204(161-288)
		23-129	12	16			
		>129	62	82			
2.	Ca (mmol/l)	<2.15	2	2.7	2.08 ± 0.021		
		2.15-2.5	73	97.3			
3.	P (mmol/l)	<0.81	34	45.3	1.13 ± 0.19		
		0.81-1.45	41	54.7			
4.	Vitamin D (ng/ml)	<20	66	88	15.05 ± 4.67		
		20-29	9	12			

ALP: alkaline phosphatase, Ca: calcium, P: phosphorous, IQR: inter quartile range

5.4. Prevalence of vitamin D deficiency and insufficiency among study participants

Vitamin D Deficiency is defined as a 25-OH D level of less than 20 ng/ml and vitamin D insufficiency as 25-OHD of 20-29 ng/ml. Among the study participants with nonspecific musculoskeletal pain symptoms 66(88%) [Male 19(25.33%) and female 47(62.67%)] were with Vitamin D deficiency and 9(12%) [Male 5(6.67%) and female 4(5.33%)] were vitamin D insufficient.

5.5. Comparison of mean of biochemical parameters between vitamin D deficient and vitamin D insufficient groups of study participants

There was no statically significant difference between the vitamin D deficient and sufficient groups in their ALP, Ca and P levels ($p > 0.05$) (Table 4).

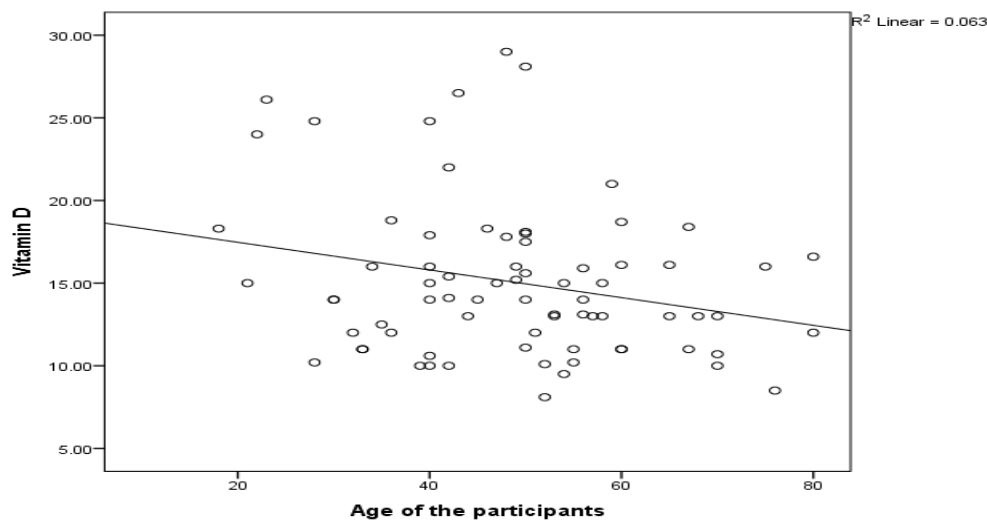
Table 4: Comparison of biochemical parameters of study participants between vitamin D deficient and vitamin D insufficient groups among patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May, 2018 (n=75)

	Parametric values	Vitamin deficient	Vitamin insufficient	P value
		group(N=66)	group(N=9)	
		Mean ± SD	Mean ± SD	
1.	P (mmol/l)	1.14 ± 0.194	1.10 ± 0.19	0.591
2.	Ca	2.09 ± 0.21	2.0544 ± 0.17	0.67
	Non Parametric values	Median	Median	P value
3.	ALP	206.50	204.0000	0.546

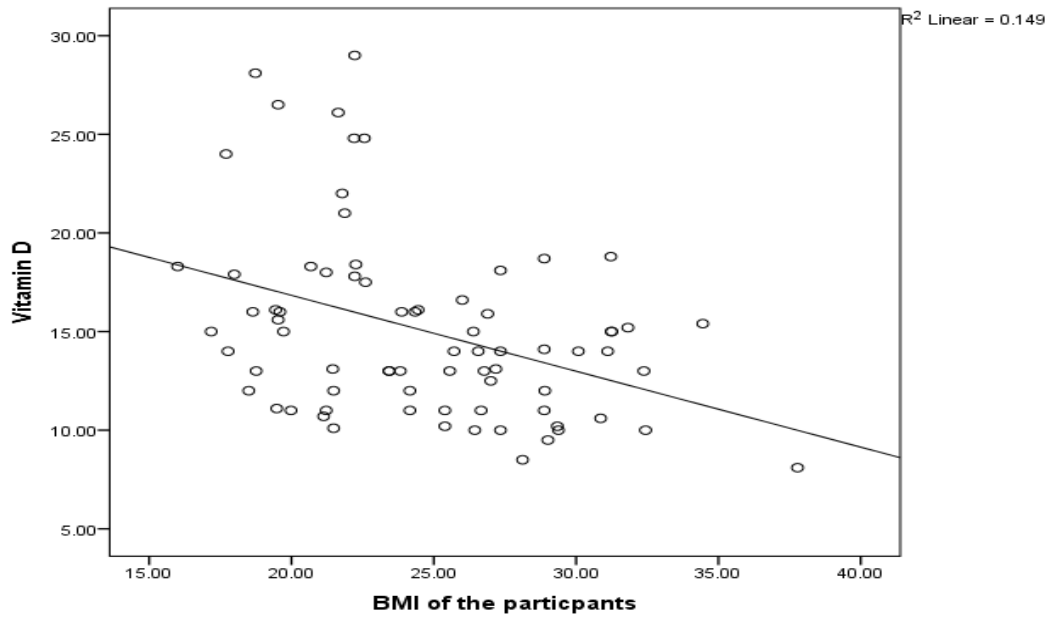
P: phosphorus, Ca: calcium and ALP: alkaline phosphatase

5.6. Correlation and multiple logistic regression analysis result of independent variables with vitamin D deficiency

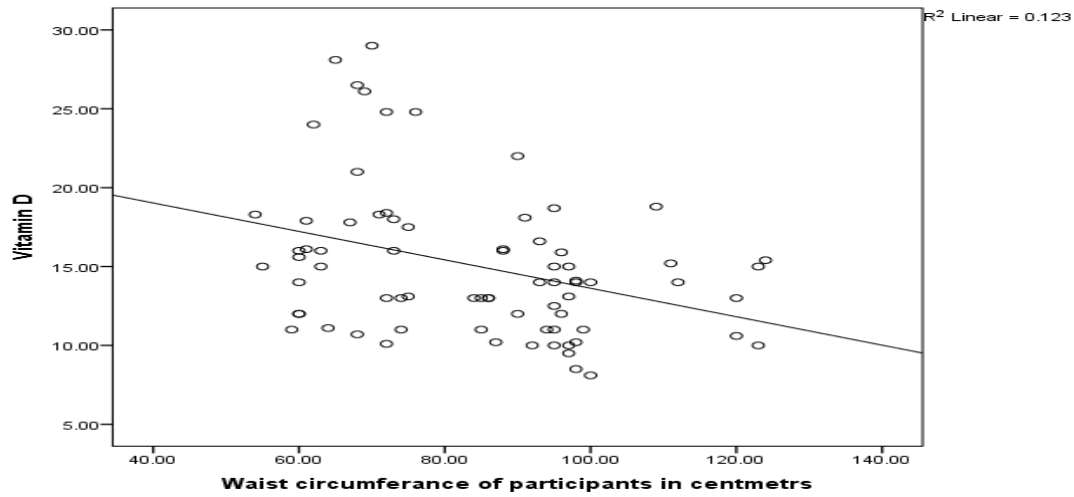
The bivariate correlation analysis showed Age ($r = -0.251$, $p=0.030$), BMI ($r = -0.388$, $P=0.001$) and WC ($r = -0.35$; $p=0.002$) were inversely correlated with Vitamin D level (**figure.5**).



A.



B.



C.

Figure 3: Scatter Plots that show relationship between vitamin D level and Age (A), BMI (B) and WC (C)

Simple logistic regression result showed only age (COR=1.063, p=0.037), occupation (COR=5.455, P=0.046), BMI (COR=1.302, p=0.001) and WC (COR=1.059, p=0.002) were significantly associated with vitamin D deficiency (**Table 5**).

Table 5: Binary logistic regression of demographic and anthropometric variables with vitamin D deficiency among patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May, 2018 (n=75)

No.	Variables	Vitamin D deficient (N=66)	Vitamin D insufficient (N=9)	COR	95% CI	P value	
1.	Age(years)	66	9	1.063	1.00-1.13	0.037*	
2.	Gender	Male	19	5	Ref.		
		Female	47	4	3.092	0.75-2.78	0.119
3.	Menopause stage	Yes	29	2	1.611	0.21-2.45	0.648
		No	18	2	Ref.		
4.	Level of education	Illiterate	9	1	2.4	0.23-4.96	0.464
		primary school	26	2	3.45	0.57-1.23	0.179
		Secondary school	16	2	2.133	0.34-13.4	0.419
		College/University	15	4	Ref.		
5.	Occupation	Farmer	2	0	-	-	
		Government employee	20	2	5.455	0.94-1.75	0.059
		Private business	11	6	Ref.		
		Unemployed	6	0	-	-	
		Housewife	18	1	9.818	1.04-2.78	0.046*
		Retired	9	0	-	-	
6.	Monthly income(ETB)	66	9	1	0.23-4.38	0.684	
7.	Household size	66	9	1.143	0.262-1.621	0.859	
8.	BMI (Kg/m ²)	66	9	1.302	1.05-1.62	0.001*	
9.	WC	66	9	1.059	1.005-1.15	0.002*	

*statistically significant difference, COR: crud odds ratio, ETB: Ethiopian birr, BMI: body mass index, WC: waist circumference

Table 6: Binary logistic regression analyses result of life style and clinical data with vitamin D deficiency among patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May, 2018 (n=75)

No.	Variables Categories	Vitamin D deficient (N=66)	Vitamin D insufficient (N=9)	COR	95% CI	P value	
1.	Time of sun exposure(hours)	66	6	0.802	0.62-1.04	0.095	
2.	Use of sun screen	Yes	3	0	Ref.		
		No	63	9	0.00	0.00	0.999
3.	Fish intake/month	Never	45	8	Ref.		
		<1	16	1	2.84	0.33-24.56	0.342
		1	4	0	-	-	
		>1	1	0	-	-	
4.	Egg intake/week	Never	29	2	Ref.		
		<1	20	3	0.46	0.07-3.00	0.417
		1	9	3	0.21	0.03-1.44	0.111
		>1	8	1	0.55	0.04-6.89	0.644
5.	Cigarette smoking habit	Yes	6	0	-	-	
		No	60	9	Ref.		
6.	Alcohol consumption habit	Yes	23	3	1.3	0.29-5.67	0.727
		No	40	6	Ref.		
7.	Duration of the pain	66	9	0.982	0.87-1.109	0.772	
8.	Other co-morbidity	No	34	8	Ref.		
		HIV AIDS	4	0	-	-	
		Hypertension	14	1	3.294	0.38-28.85	0.282
		DM	10	0	-	-	
		Others	4	0	-	-	

COR: crud odds ratio, HIV AIDS: human immune virus acquired immunodeficiency syndrome, DM: diabetes mellitus

Table 7: Binary logistic regression analyses result of biochemical parameters with vitamin D deficiency among patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May, 2018 (n=75)

No.	Variables Categories	Vitamin D deficient (N=66)	Vitamin D insufficient (N=9)	COR	95% CI	P value
1.	ALP	66	9	1.003	0.99-1.01	0.32
2.	Ca	66	9	1.517	0.06-97.21	0.62
3.	P	66	9	2.879	0.03-246.2	0.641

COR: crude odds ratio, ALP: alkaline phosphatase, Ca: calcium, P: phosphorous

Finally, multivariate model with forward Wald elimination showed only BMI (AOR=1.302 p=0.018) was remained as independent risk factor of vitamin D deficiency. One kg/m² increase in BMI increase odds of developing vitamin D deficiency by 1.302 times. (Table 8)

Table 8: Multiple logistic regression analyses result of predictor of vitamin D deficiency among patients with nonspecific musculoskeletal pain symptoms at neurology clinic of TASH from March to May, 2018

No.	Variables	Vitamin D deficient (N=66)	Vitamin D insufficient (N=9)	AOR	95% CI	P value	
10.	Age(years)	66	9	1.063	1.00-1.13	0.107	
11.	Gender	Male	19	5	Ref.		
		Female	47	4	3.092	0.75-2.78	0.139
12.	Level of education	Illiterate	9	1	2.4	0.23-4.96	0.564
		primary school	26	2	3.45	0.57-1.23	0.189
		Secondary school	16	2	2.133	0.34-13.4	0.489
		College/University	15	4	Ref.		
13.	Occupation	Farmer	2	0	-	-	
		Government employee	20	2	5.455	0.94-1.75	0.129
		Private business	11	6	Ref.		
		Unemployed	6	0	-	-	
		Housewife	18	1	9.818	1.04-2.78	0.086
		Retired	9	0	-	-	
14.	BMI (Kg/m ²)	66	9	1.302	1.05-1.62	0.018*	
15.	WC	66	9	1.059	1.00-1.15	0.062	

* Statistically significant association, AOR: adjusted odds ratio, BMI: body mass index, WC: waist circumference, CI: confidence interval

6. Discussion

We assessed Vitamin D deficiency and its associated risk factors among 75 Ethiopian participants with nonspecific musculoskeletal pain symptoms attending treatment follow up at TASH. It was the first study performed in this population at this tertiary setting aiming to determine prevalence of Vitamin D deficiency and its associated risk factors and compared differences in biochemical parameters in Vitamin D deficient and Vitamin D insufficient groups of this population.

Prevalence of Vitamin D deficiency

In this study all the participants had vitamin D level below normal (<30 ng/ml). Among these participants with nonspecific musculoskeletal pain symptoms 66(88%) [Male 19(25.33%) and Female 47(62.67%)] were with Vitamin D deficiency and 9(12%) [Male 5(6.67%) and female 4(5.33%)] were vitamin D insufficient. Dark skin might be the reason for the relatively high vitamin D inadequacy in this study. Melanin is known to block UVB radiation(73). Other possible reason might be due to eating diet poor in vitamin D among Ethiopians(13).

A similar cross sectional study conducted in USA on patients with persistent nonspecific musculoskeletal pain symptoms with age ranging from 10 to 60 years reported consistent prevalence (93%) to our study(38). Another study with smaller sample size of 62 in Iran on adult patients with nonspecific musculoskeletal pain symptoms reported comparable prevalence of 95.4 % (46). A case control study done in Pakistan on 60 cases with nonspecific musculoskeletal pain symptoms and 60 controls showed consistent prevalence of vitamin D among cases (86.6%) (50).

A cross sectional studies done in Pakistan(48, 49) with small sample sizes of 40 and 100 reported slightly lower prevalence of 80%, and 72% respectively compared to our study. The possible explanation may be the study subjects' age difference between study participants. Our study participants mean age was 48.9 while the former's study mean age was 37.65 ± 11.5 and the latter's study participants were children from 10 to 12 years old. Cutaneous vitamin D synthesis declines with age(55). Another studies done in Saudi 60 patients with chronic and diffuse nonspecific musculoskeletal pain symptoms also identified slightly lower (70%) prevalence of vitamin D deficiency and 20% vitamin D insufficiency(44). A similar study in Switzerland 174 patients with chronic pain reported slightly lower (71%) prevalence of vitamin D deficiency and 21% vitamin D insufficiency(40). Unlike to our study a cross sectional study done with small sample sizes in Turkey on 145 chronic low back pain patients identified lower (22.8%) prevalence of vitamin D deficiency, 42.8% vitamin D insufficiency(43). Another study in Singapore on 114 participants showed 42% prevalence of vitamin D deficiency(57). This may be due to difference in life style, dietary habits and availability of vitamin D supplementation therapies in European countries.

A cross sectional studies conducted on large scale sample sizes as a study in Iraq reported prevalence of vitamin D deficiency 85.35% in a sample of 355 patients with chronic nonspecific musculoskeletal pain symptoms(47). Another study on 386 North Korean refugees in South Korea found prevalence of vitamin D deficiency as 87%(54). A controlled study in India reported 86% prevalence of vitamin D deficiency among 328 chronic low back pain patients(53). These findings are consistent with our study's result.

Another cross sectional studies conducted on large scale sample sizes as in studies done in Turkey(42) and Pakistan(52) reported slightly lower prevalence of vitamin D deficiency as 71.7 % and 80% respectively. This could be possibly explained by the difference in mean age between our study participants and in these studies.

However, a studies done in USA(37), India(24), Iran(11), and Saudi(45) on larger sample size found lower prevalence of 26%, 40.2%, 47% and 49.19% respectively. This may be due to difference in life style, skin color, and living condition.

Association of vitamin D level with independent variables: We observed that gender, menopause stage, level of education, use of sun screen, monthly fish intake, weekly egg intake, history of cigarette smoking, duration of cigarette smoking, number of cigarette, alcohol consumption, household size, income level, duration of pain compliant, duration of sun exposure, other co-morbidity, Ca, P and ALP were not significantly associated with vitamin D level.

In contrast to our study, a study in Bahrain(67) showed that there was association of vitamin D deficiency with high income level (P-value 0.020) and habit of smoking (P-value 0.021).

In line to our study, a study conducted in Turkey(41) showed no association of vitamin D deficiency with duration of pain compliant, level of income, level of education and duration of sun exposure. Similarly a study in Bahrain(62) showed no association of vitamin D deficiency with duration of sun exposure. In contrast to this a study in USA(59) showed vitamin D deficiency was common among those who were not collage educated (P <0.05). Another contrasting studies in Qatar(61), Bahrain(67), USA(37), India(53) and Korea(66) showed association of vitamin D deficiency with duration of sun exposure.

In our study age, BMI, WC and occupation were associated with vitamin D deficiency. In line to our study, a studies conducted in Turkey(42), India(24), Qatar(61), Iran(11, 62), Iraq(47) and USA(64) showed association of vitamin D deficiency with age. In contrast to these studies, studies in Saudi Arabia(33) and Turkey(41, 68) showed no association of vitamin D deficiency with age. It is well established that cutaneous vitamin D synthesis declines with age(55)

In line with our study, studies in South Korea(54), Singapore(57), USA(37, 56, 59, 60, 64), Bahrain(67) and Qatar(61) showed association between BMI and vitamin D deficiency. In contrast to this finding, a study in Teheran(62), Turkey(41) and Switzerland(58) showed no association of vitamin D deficiency with BMI.

In this study housewives were more vitamin D deficient compared to those who were private company owners and employers. This might be due to limited outdoor activities by housewives compared to private company owners and employers. Sedentary way of life limits the exposure of skin to UVB radiation and hence related to vitamin D deficiency(1). In line to our study a study done in Iraq(47) showed association of vitamin D deficiency with occupation. In contrast to this a study in Turkey(41) showed no association of vitamin D deficiency and occupation.

Comparison of biochemical parameters between vitamin D deficient and Vitamin D insufficient groups: In our study, P, Ca and ALP were not significantly differs between vitamin D deficient and vitamin D insufficient group.

In line to our study a study in Turkey(41) showed no difference in levels of Ca, P and ALP between those with vitamin D level of <20ng/ml and those with vitamin D level of >20ng/ml. Another study in Denmark(69) and Turkey(68) also showed no relationship between levels of Ca and P ALP and vitamin D metabolites. Similarly, a study conducted in Pakistan(49) reported no significant difference in P and Ca levels but with regard to ALP showed significantly higher activity in vitamin D deficient group.

In contrast to our study, a studies in Qatar(61) showed that level of Ca, ALP and P were low in vitamin D deficient children. Similarly a study in India(70) reported level of Ca, ALP, P and vitamin D were positively correlated.

Risk factors of Vitamin D deficiency: According to result of multiple logistic regression analysis of the association between independent variables and vitamin D deficiency, BMI was remained as independent risk factor of vitamin D deficiency. The relationship of obesity and vitamin D deficiency may be due to increased volume of distribution to fat of lipid soluble vitamin D as it leaves the general circulation after being synthesized in the skin or obtained through the diet. Moreover the vitamin also retains in those fat stores(74). Studies conducted in USA(59, 64), Singapore(57) and Bahrain (67) reported BMI as independent risk factor of vitamin D deficiency.

7. Strength and limitations of the study

7.1. Strength of the study

- To our knowledge this study is new among our study participants with nonspecific musculoskeletal pain symptoms.
- Moreover, Ca and P levels were determined in accredited referral clinical chemistry of Ethiopian Public Health Institute.

7.2. Limitations of the study

- Convenient sampling technique was used to select the study participants
- This study was conducted on small sample size in a tertiary referral neurology clinic where most of the patients had many complications and numerous co morbidities. Because of this fact, results of this study cannot therefore be generalized to the majority of patients with nonspecific musculoskeletal pain symptoms who follow up at primary health care.
- Some of the information like the use of sun screen, fish intake, egg intake, hours of sun exposure, habits of smoking and alcohol consumption were subjectively obtained from patients.
- Total ALP in serum was analyzed without identifying bones specific ALP.
- Moreover, our inability to measure PTH which is a better bone mineral density indicator with ALP, Ca and P due to unavailability of these assays and cost.

Conclusions

In conclusion, this cross sectional study indicated the Prevalence of vitamin D deficiency as 88% and vitamin D insufficiency as 12%.

Age, BMI, WC and occupation are associated with vitamin D deficiency. This study demonstrated that obesity is independent risk factor for vitamin D deficiency.

Recommendations

Based on our findings, we would like to put the following recommendation:

1. Patients with nonspecific musculoskeletal pain symptoms need to have evaluation of Vitamin D levels in order to have a better picture about their disease status.
2. Further study should be conducted to:
 - Establish the local reference range of vitamin D level for Ethiopians, and then the prevalence of vitamin D deficiency and associated risk factors among patients with nonspecific musculoskeletal pain symptoms should be studied based on the established reference range.
 - Determine the association of vitamin D with biochemical marker by testing the participants serum for PTH and bone specific ALP
 - Determine the effect of vitamin D supplementation therapy on pain symptoms among patients with patients with nonspecific musculoskeletal pain symptoms.

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Annexes

Annex I: Information sheet

A. English version of participant information sheet and informed consent.

Addis Ababa University College of Health Sciences Department of Medical Laboratory Sciences

Participant information sheet

You are invited to participate in a study to be conducted by MSc student Amanuel Dubale at Addis Ababa University, College of Health Sciences and Department of Medical Laboratory Science. Please read the following statements and ask any unclear points before you agree to participate.

Introduction: The topic of this study is “Prevalence of Vitamin D deficiency and associated risk factors in Patients with nonspecific musculoskeletal pain symptoms attending Tikure Anbesa Specialized Hospital”. Participation in this study is exclusively voluntarily. If you are not interested to participate, there will be no consequences.

What is expected from me as participant of the study?

As a participant of this study, you will be asked to give 5ml of blood sample, which will be used to determine serum Vitamin D, PTH, Calcium, ALP and P. You will also let us measure your waist circumference, weight and height. You are also expected to respond to questions included in the questioner and interviews which will be used as relevant data for this particular research.

Potential benefits to participant and/ or to the society

1. You will get laboratory test result of total Vitamin D, PTH and Ca, P and ALP which are costly for free.
2. The study will determine prevalence of Vitamin D deficiency and knowing prevalence is important to the clinician to look for vitamin D deficiency in patients with nonspecific musculoskeletal symptoms and carryout appropriate management. In case you will be vitamin D deficient the result will be reported to your attending clinician for appropriate management.
3. It will also identify major risk factors for vitamin D deficiency which correlate to nonspecific musculoskeletal symptoms to facilitate prevention, early diagnosis, and early treatment as some of them are modifiable.

4. Additionally the study will recommend inclusion of these analytes (Vitamin D, PTH, Ca, P and ALP) in a routine test for patients with nonspecific musculoskeletal symptoms presenting symptoms of vitamin D deficiency. Hence, you are directly or indirectly benefiting yourself, other patients and the society at large.

Compensation for participation: You will not receive any payment for your participation in this research study.

Confidentiality: On the request paper your name or your identities will not be mentioned.

Samples and information given by the participants will serve only for this research not for any other purpose.

Person to contact: Please direct any questions you may encounter during this study to the principal investigator.

Amanuel Dubale: Email: zamuyeeadk@gmail.com or mobile +251916702777

Department of Medical Laboratory Science research ethics office +251 11 275 5170

Code No. _____

Consent form

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction.

I voluntarily consent that I would participate in this study to collect my blood and be a participant in this study and understand that I have the right to withdraw from the study at any time.

Print name of participant, date and signature or thumb impression of participant

_____ /____ /____ (dd/mm/yy) _____

If illiterate;

Print name of independent literate witness, date and signature of witness (if possible, this person should be selected by the participant and should have no connection to the research team)

_____ /____ /____ (dd/mm/yy) _____

Phone number _____

Print name of researcher, date and signature of researcher

_____ /____ /____ (dd/mm/yy) _____

B. የተሳታፊዎች መረጃ መሰጫ እና የሰምምነት መጠየቂያ ቅጽ

በአዲስ አበባ ዩኒቨርሲቲ፤ የጤና ሳይንስ ኮሌጅ የህክምና ላቦራቶሪ ትምህርት ክፍል በሁለተኛ ዲግሪ ተማሪ አማካኝነት ጸሐፊ የሚካሄደውን የመመረቂያ ጥናት ላይ እንድሳተፉ ተጋብዘዋል። እባክዎ በዚህ ጥናት ላይ ከመሳተፍዎ በፊት ከዚህ ቀጥሎ የሚገኘውን ጽሁፍ በጥሞና ያንብቡና ይመልሱ፤ ግልጽ ያልሆነ ነገር ካጋጠሞት ይጠይቁ።

መግቢያ

የጥናቱ ርዕስ “ በጥቁር አንበሳ ስፕሻላይዝድ ሆስፕታል ካሉ የአጥንት ና ጡንቻ ህመምተኞች ውስጥ የሻታሚን ዲ ማነስ እና ተያያዥ አስጊ ሁኔታዎችን ይገመግማል”። እርስዎ በዚህ ጥናት ላይ የሚኖሩት ተሳትፎ ሙሉ በሙሉ በበጎ ፈቃዳኝነት ላይ የተመሰረተ ነው። በዚህ ጥናት ውስጥ ላለመሳተፍ ከወሰኑ በዚህ የህክምና ቦታ ውስጥ የሚሰጥዎት አገልግሎት አይቋረጥም። በጥናቱ ለመሳተፍ የሚሰማሙ ከሆነ የሰምምነት ቅጹ ላይ በጽሁፍ ወይም በጣት አሻራ ፊሩማዎችን ማስቀመጥ ይጠበቅቦታል።

የጥናቱ ተሳታፊ በመሆን ምን ይጠበቅብናል?

በዚህ ጥናት ውስጥ ለመሳተፍ ፍቃደኛ ከሆኑ 5 ሚሊ (አንድ የሻይ ማንኪያ የሚሆን) የደም ናሙና የሰጣሉ። ይህም የደም ናሙና በደም ውስጥ የሚገኘውን የሻይታሚን ዲ ፣የካልሲየም ና የፎስፈረስ መጠን እንዲሁም የአላካላይን ፎስፈረስ እንዲሁም እንቅስቃሴ በላቦራቶሪ ለመመርመር ይረዳል። የወገብ ሰፋት፣ ቁመት እና ክብደትዎን እንዲለካ ይጠየቃል። ለጥናቱ ምረጃ የሚሰጡ በመጠየቁ ውስጥ የተካተቱ ጥቂዎች ተክክለኛውን መረጃ ይሰጣሉ።

በዚህ ጥናት ውስጥ መሳተፍ የሚያስገኛቸው ጥቅሞች

1. የላቦራቶሪ ምርመራ የሻይታሚን ዲ፣ የካልሲየም እና የፎስፈረስ መጠን እንዲሁም የአላካላይን ፎስፈረስ እንዲሁም እንቅስቃሴ ጥናቱ ሲያልቅ በነጻ ያገኛሉ።
2. የሻይታሚን ዲ ማነስ ጥናቱ ስለሚጠቁም ህኪሞች ችግሩን ትኩረት ሰጥተው እንድያዩ ና የተከታታይ ህኪምና አካል አድርገው መፍትሄ እንድፈልጉላት መረጃ ይሰጣል።
3. ከዚህ የሻይታሚን ዲ ማነስ ጋር ተያያዥነት ያላቸው አስጊ ሁኔታዎችን ለይቶ ሳይባሉ ለመከላከል እና ለማከም ይረዳል።
4. ጥናቱ የሻታሚን ዲ ማነስ ላለባቸው የአጥንት እና የጡንቻ ህመምተኞች በህኪምና ክትትላቸው ወቅት የሻይታሚን ዲ፣የካልሲየም ና የፎስፈረስ መጠን እንዲሁም የአላካላይን ፎስፈረስ እንዲሁም እንቅስቃሴ እንድለካላቸው ለሆስፕታሉ ይጠቁማል። ስለዚህ በጥናቱ በመሳተፍዎ በቀጥታም ሆነ በተዘዋዋሪ መንገድ ለራሱ፣ ለሌሎች ህመማን ብሎም ለማህበረሰቡ ይጠቅማሉ ማለት ነው።

በዚህ ጥናት ላይ በመሳተፍዎ የሚከፍሉትም ሆነ ሚከፈሉት ክፍያ የለም።

የተሳታፊዎችን ምስጢር ስለመጠበቅ፤ በመጠየቂያው ወርቀት ላይ የተሳታፊዎች ስም ወይም ማንነት አይገለጽም ። በተሳታፊዎች የሚሰጥ ናሙና ለዚህ ጥናት ጥቅም ብቻ የሚያገለግል ይሆናል።

ጥያቄ ካሉት ፤ ይህ ጥናት በተመለከተ ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥሙ ድንገተኛ ችግሮች ወይም ጥያቄ በሚከተለው አድራሻ ይጠቁሙ።

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የስምምነት መጠየቂያ ቅጽ

ኮድ. ቁ. _____

ከላይ የተሰጠውን መረጃ አንብቢያለሁ ወይም ተነባኛል። ጥያቄን እንድጠይቅ እድል ተሰጥቶ ጠይቄ አርኪ መልስ ተመልሶልኛል።

ዋናቱ ላይ ተሳትፈ የደም ናሙና እና አስፈላጊውን መረጃ ለመስጠት በፍቃድ እየተሰማማሁ በማንኛውም ሰዓት ከዋናቱ ለመውጣት መብት እናዳለኝ አረጋግጣለሁ።

የተሳታፊው ስም _____

ቀን ____ / ____ / ____

ፊሩማ ወይም የጣት አሻራ _____

ያልተማሩ እንደሆነ

የተማረ ሰው የአይን ምስክር _____

ቀን ____ / ____ / ____

ፊሩማ _____

ስ.ቁ _____

የአዋኝው ስም _____

ፊሩማ _____

ስ.ቁ _____

B: የአግርኛ ቃለ መጠየቅ

ኮድ _____

እባክዎ አጭር መልስ ለሚረዱት ጥያቄዎች መልስዎን ይጻፉ ። ምርጫ ላላቸው ደግሞ የመረጡትን መልስ ያክብቡ።

Part Ib: የተሳታፊዎች አጠቃላይ መረጃ

1. ዕድሜዎት ስንት ነው? _____
2. ጾታ ሀ. ወንድ ለ. ሴት
3. ሴት ከሆኑ
እርግት ደረሰዋል? ሀ. አዎ ለ. አይደለም
4. የትምህርት ደረጃ? ሀ. ያልተማረ/ች ለ. አንደኛ ደረጃ ሐ.ሁለተኛ ደረጃ መ. ኮልጅ/የኒቨርሲቲ
5. የስራ ሁኔታ? ሀ. ገበረ ለ. የመንግሥት ሰራተኛ ሐ.የግል ድርጅት መ. የቤት እመቤት ሠ. ስራ አጥ
6. የወር ገቢ _____
7. የቤተሰብ ቁጥር _____

Part IIb: ለሻይታሚን ዲ ማሰ አሰጪ ሁኔታዎችን ለመለየት የሚረዱ ጥያቄዎች

1. በቀን ምን ያክል ሰዓት ጸሀይ ያገኛሉ? _____
2. ጸሀይ ለመከላከል የሚቀጣ ቅጣት ይጠቀማሉ? ሀ. አዎ ለ.አይደለም
3. በወር ስንተ ዓሳ ይመጣሉ? _____
4. በሳምንት ስንተ እንቁላል ይመጣሉ? _____
5. ስካራ አጭሰው ያውቃሉ? ሀ. አዎ ለ. አይደለም ሐ.አቁሚያለሁ
6. ካጨሰሱ ለምን ያህል ጊዜ(በአመታት)? _____
7. ካጨሰሱ በቀን ምን ያህል ስካራ ያጨሳሉ? _____
8. አልኮል ይጠጣሉ? ሀ. አዎ ለ. አይደለም ሐ.አቁሚያለሁ

Annex III: Data collection checklists

A. Checklist to gather medical history and current medication from medical records

No.	Parameter	Record	Remark
1.	Duration of compliant in months		
3.	Other co-morbidity	Diabetes mellitus_____	
		Autoimmune diseases(specify)_____	
		Other(specify)_____	

B. Checklist to record anthropometric data

No	Variables	Value	Remark
1.	Body weight		
2.	Height		
3.	BMI		
4	Waist circumference		

C. Checklist to record laboratory findings

No	Laboratory parameters	Value	Remark
1.	Vitamin D(OH-25)		
3.	Ca		
4.	P		
5.	ALP		

Annex V. Standard operating procedures (SOP)

A. Sample collection

Take blood samples from the anti-cubital vein of the arm by using syringes after proper antisepsis with alcohol and sterile cotton swabs. Then transfer the blood from each participant to serum separator tube and allowed to stand for 30 minutes. Separate serum by centrifugation at 1500 rpm. Follow safety precaution.

Procedure for serum separation

1. Drawn 5 ml whole blood into serum separator tube containing no anticoagulant.
2. Kept in upright position at room temperature for 30-45 min to allow clotting.
3. Centrifuged for 5 min at manufacturer's recommended speed.
4. Carefully aspirate the serum at room temperature and pool into a centrifuge tube, taking care not to disturb the cell layer or transfer any cells. Use clean pipette for each tube.
5. Inspected serum for turbidity. Centrifuge turbid samples and aspirate again to remove remaining insoluble matter.
6. Separate serum sample
7. Aliquot into nunc tubes and stored at -20°C . Label nunc tubes with patient identification number.

B. SOP for Vitamin D determination

Clinical relevance

This assay is intended for the quantitative determination of total vitamin D (25-OH) in human serum and plasma, as an aid in the assessment of vitamin D sufficiency in patients with bone disorders, in children with rickets, in elderly with risk of falling, diabetes, cancer, cardiovascular disease, and autoimmune diseases.

Test principle and procedure of vitamin D determination

Test principle: the assay principle combine an enzyme immunoassay competition method with a final fluorescent detection (ELFA). The solid phase receptacles (SPR) serve as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready to use and pre dispensed in the sealed reagent strips. All the assay steps are performed automatically by the instrument. The reaction

medium is cycled in and out of the SPR several times. The sample is mixed with pretreatment reagent to separate vitamin D from its binding protein. The pretreated sample is then collected and transported into the well that contains an alkaline phosphatase (ALP) labeled anti vitamin D antibody conjugates. The vitamin D antigen in the sample and the vitamin D antigen coated in the interior of the SPR compete for binding site on the anti-vitamin D antibody-ALP conjugate.

During final detection step, the substrate (4-Methyl-umberferyll phosphate) is cycled in and out of the SPR. The conjugate enzyme catalysis the hydrolysis of this substrate in to a fluorescent product, 4-Methyl-umberferone, the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of vitamin D antigen present in the sample. At the end of the assay, results are automatically calculated by the instrument in relation to the calibration curve stored in the memory, and then print out.

Sample: human serum or lithium heparin plasma

Sample preparation: after thawing frozen sample were homogenized by vortex mixer before testing. Samples with fibrin clot or erythrocyte stroma were centrifuged before testing.

Procedure

1. Remove one VITD strip and one VITD SPR for each sample, control and calibrator to be tested.
2. The test is identified by the VITD code on the instrument. The calibrator must be identified by S1 and tested in duplicate and the control by C1.
3. Mix the calibrator, control and samples using vortex type mixer for serum or plasma separated from pellet.
4. Add 100 ul of calibrator, control and sample in to the VTID reagent strip.
5. Before pipetting make sure that the sample, control and calibrator are free of bubbles.
6. Insert the VITD SPR and VITD strips in to the instrument. Check to make sure the color abeles with the assay code on the SPRs and the reagent strips match.
7. Initiate the assay, the assay steps are performed automatically by the instrument.
8. Reclose the vials and return them to 2-8⁰c after pipetting.
9. The assay will be finished within approximately 40 minutes. After the assay is completed remove the SPRs and strips from the instrument and dispose of in to appropriate recipient.
10. Results are determined via an instrument-specific calibration curve which is generated by 2-point calibration and a calibration master curve provided via the reagent barcode.

Limitation of the assay: interference may be encountered with certain sera containing antibodies directed against reagent components.

Range of expected values: vitamin D deficient (<20ng/ml), vitamin D insufficient (20-29ng/ml), vitamin D sufficient (30-100ng/ml) and vitamin D potential toxicity (>100ng/ml).

C. SOP for ALP determination

Clinical relevance: Serum alkaline phosphatase estimations are of interest in the diagnosis of two groups of conditions; hepatobiliary disease and bone disease associated with increased osteoblastic activity.

Test principle and procedure of ALP determination

Test principle:



P-Nitro phenyl phosphate is hydrolyzed to p-nitro phenol and inorganic phosphate. The rate at which the p-NPP is hydrolyzed, measured at 405 nm, is directly proportional to the alkaline phosphatase activity.

Specimen: Serum samples should be stored at 2-8°C and run within two days.

Reference range:

Adults

Males 40-129 IU/L Adults 35-123 IU/L

Limitations:

1. This methodology measures total alkaline phosphatase irrespective of tissue or organ of origin. Further tests may be necessary to assist in differential diagnosis.
2. Samples with values exceeding 1000 IU/L should be diluted with an equal volume of saline and re-assayed multiplying the results by two.

D. SOP for Ca determination

Clinical relevance: Serum calcium levels and hence the body content are controlled by parathyroid hormone (PTH), calcitonin, and vitamin D. An imbalance in any of these modulators leads to alterations of the body and serum calcium levels. Increases in serum PTH or vitamin D are usually associated with hyperkalemia. Increased serum calcium levels may also be observed in multiple myeloma and other neoplastic diseases. Hypocalcaemia may be observed in hyperparathyroidism, nephrosis, and pancreatitis.

Test principle and procedure of Ca determination

Calcium ions react with 5-nitro-5'-methyl-BAPTA (NM-BAPTA) under alkaline conditions to form a complex. This complex reacts in the second step with EDTA. The change in absorbance is directly proportional to the calcium concentration and is measured photo metrically at 340 nm.

Specimen: Serum or heparinized plasma

Reference range:

Serum/plasma: 2.15-2.5 nmol/l

E. SOP for P determination

Clinical relevance: Phosphorus occurs in blood in the form of inorganic phosphate and in organically bound phosphoric acid. The small amount of extracellular organic phosphorus is found almost exclusively in the form of phospholipids. The ratio of phosphate to calcium in the blood is approximately 6:10. An increase in the level of phosphorus causes a decrease in the calcium level. The mechanism is influenced by interactions between parathyroid hormone and vitamin D. Hyperparathyroidism, vitamin D intoxication and renal failure with decreased glomerular phosphate filtration give rise to hyperphosphatemia. Hypophosphatemia occurs in rickets, hyperparathyroidism and Fanconi's syndrome.

Test principle and procedure of P determination

Test principle: Molybdate UV.

Procedure of P determination

1. Inorganic phosphate reacts with ammonium phosphomolybdate complex having the formula $(\text{NH}_4)_3[\text{PO}_4 (\text{MoO}_3)_2]$ with ammonium molybdate in the presence of sulfuric acid.
2. The concentration of phosphomolybdate formed is directly proportional to the inorganic phosphate concentration and is measured photometrically.

Reference range: 0.81-1.45nmol/l

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Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

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Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

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Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: Tatek Gebreegzeabeher (MSc, PhD candidate)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.