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Assessments of Serum level Vitamin D and Thyroid Function Tests among Newly Diagnosed Female Breast Cancer Patients attending in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Mehari Meles Lema, entitled:

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Abbreviation

BC	Breast Cancer
BMI	Body mass index
Ca	Calcium Total
E2	Estradiol
ECLIA	Electrochemiluminescenceimmunoassay
EPHI	Ethiopian Public Health Institute
FT3	Free Triiodothyronine
FT4	Free Thyroxine
LC/MS/MS	Liquid chromatography coupled with tandem mass spectrometry
SPSS	Statistical Package for the Social Sciences
SVDD	Severe Vitamin D Deficiency
Prog	Progesterone
PRL	Prolactin
Phos	Phosphate
TASH	Tikur Anbessa Specialized Hospital
TH	Thyroid Hormones
TRH	Thyroid-Releasing Hormone
TSH	Thyroid-stimulating hormone
TT3	Total Triiodothyronine
TT4	Total Thyroxine
WHO	World Health Organization
VDD	Vitamin D Deficiency

Abstract

Background: Breast cancer (BC) is the most common cancer in women, with high global mortality. Vitamin D deficiency and thyroid dysfunction may influence BC through hormonal regulation and immune modulation. Thyroid hormones affect cell metabolism and proliferation, while vitamin D has anti-proliferative effects on breast tissue. Thyroid dysfunction can alter estrogen metabolism, impacting BC risk. However, data on serum vitamin D and thyroid hormone levels in Ethiopian BC patients are limited. This study aims to assess these levels in newly diagnosed female BC patients.

Methods: A Facility-based comparative cross-sectional study was conducted from January to March 2024 in Tikur Anbessa Specialty Hospital. A convenient sampling method was employed to recruit 69 females newly diagnosed with breast cancer as a case group and 69 samples from apparently healthy females as a control group. Blood samples were collected and sent to Ethiopian Public Health Institute (EPHI) for Serum 25 hydroxyl vitamin D (25(HO) D) Concentration and Thyroid Function Tests , by using a COBAS 6000 analyzer. The data was analyzed using SPSS version 20.0 software. Independent T-tests, chi-square test, One-way ANOVA tests, Kruskal Wallis test, and multiple linear regression tests were used to analyze the data.

Results: Breast cancer patients had significantly lower Total Triiodothyronine (TT3) levels (1.2±0.28 ng/mL) than healthy controls (1.4±0.19 ng/mL, $p<0.001$). Free Triiodothyronine (FT3) was also significantly reduced in BC patients ($p< 0.001$). 25(HO) D levels showed a significant lower median value in BC patients compared to healthy control groups ($p=0.043$). Breast cancer stage and subtype significantly influence thyroid function and vitamin D levels, with advanced stages associated with altered thyroid hormones and reduced 25(OH) D levels.

Conclusion: Breast Cancer patients exhibited significantly lower TT3 and FT3 levels compared to controls. Although vitamin D deficiency was prevalent in both groups, severe deficiency (<10 ng/mL) was significantly higher in the BC patients. Therefore, monitoring and managing vitamin D and thyroid hormone levels could be important components of breast cancer risk assessment and prevention strategies. However, larger-scale studies are needed to further validate and strengthen these findings.

Keywords: Breast Cancer, Thyroid Hormones, TSH, 25(OH) D, Vitamin Deficiency

1. Introduction

1.1 Background

Breast cancer (BC) is one of the most ancient and prevalent malignancies, first described by the Egyptians over 3000 years ago. While BC affects both men and women, it is approximately 100 times more common in women and remains the most frequently diagnosed cancer among them. Globally, it accounts for one in ten new cancer cases annually and is the second most common cancer overall, following lung cancer. Despite its prevalence, BC in males has lower survival outcomes due to delayed diagnosis (1–3).

BC typically originates in the ducts (85%) or lobules (15%) of the breast gland. In its earliest stage (carcinoma in situ), the disease is confined within the ducts, posing minimal risk of metastasis. However, over time, in situ tumors can become invasive, spreading to nearby tissues, regional lymph nodes, or distant organs such as the lungs, liver, bones, or brain (4). BC progresses from Stage 0 (in situ) to Stage IV, with higher stages indicating more extensive metastasis. Mammography remains the gold standard for screening and diagnosing localized BC, while serum biomarkers such as carcinoembryonic antigen (CEA) and CA15-3 are useful for monitoring metastatic disease progression (5,6).

Several risk factors contribute to BC development, including age, gender, family history, reproductive hormones, smoking, late menopause, and delayed first full-term pregnancy. Genetic predispositions, such as mutations in the BRCA1, BRCA2, and Ataxia Telangiectasia Mutated (ATM) genes, also elevate BC risk due to increased radiation sensitivity (7,8). Additionally, increased body mass index (BMI) is strongly linked to BC, particularly in postmenopausal women, as excess adipose tissue promotes estrogen production. Elevated estrogen levels can fuel estrogen receptor-positive BC growth (9). Conversely, protective factors against BC include reduced alcohol, fat, and red meat intake, as well as increased dietary fiber and vitamin D (10).

Hormonal regulation plays a crucial role in BC development. Breast tissue growth and differentiation are influenced by several hormones, including prolactin (PRL), estrogen (E2), progesterone (Prog), insulin, and thyroid hormones (TH) (11,12). E2 stimulates tumor growth, while Prog, in controlled amounts, has a protective effect. PRL dysregulation has also been implicated in increased BC risk (13–15).

The hypothalamic-pituitary-thyroid (HPT) axis influences BC pathogenesis through direct effects on tumor cells and indirect systemic alterations. Thyroid dysfunction is increasingly recognized as a contributing factor to BC (16,17). Hyperthyroidism, both overt and subclinical, has been associated with an increased incidence of BC and other malignancies. Thyroid-stimulating hormone (TSH), which regulates T4 and T3 production, may also influence BC pathogenesis by modulating cellular proliferation (18). Thyroid disorders, including hypothyroid ism, hyperthyroidism, goiter, and thyroid cancer, are particularly prevalent in women, further reinforcing the need to examine thyroid function tests in BC patients (19).

Vitamin D, first identified by Edward Mellan in 1919 for its role in preventing rickets, is a lipid-soluble molecule essential for calcium (Ca) and phosphate (Phos) homeostasis (20). Emerging evidence suggests that vitamin D also plays a role in BC prevention and progression. Low vitamin D levels have been associated with increased BC risk, particularly in individuals with high BMI, advanced age, limited sunlight exposure, high latitude, and dark skin pigmentation. Vitamin D, along with parathyroid hormone (PTH), regulates Ca and Phos metabolism, which influences BC development and progression, particularly in cases of bone metastasis. Altered mineral levels in BC patients may further contribute to tumor progression (21,22).

The interplay between BC, thyroid function, and vitamin D is complex but clinically significant. Thyroid hormones can promote BC progression through their mitogenic effects, while vitamin D may counteract these influences by exerting anti-proliferative and pro-apoptotic effects on BC cells. Understanding these interconnections is essential for optimizing patient management, as evaluating thyroid profiles and vitamin D status in BC patients may provide valuable insights for risk assessment, prognosis, and targeted interventions. This study assessed thyroid profile tests and vitamin D levels in newly diagnosed breast cancer patients at Tikur Anbessa Specialized Hospital (TASH), Addis Ababa, Ethiopia.

1.2. Statements of the Problem

BC was the majority of commonly diagnosed cancer in 2020, accounting for nearly 12% of all cancer cases, 2.3 million women were diagnosed with 685,000 deaths. The disease can also arise in males although these cases represent less than 1% of all cancer cases worldwide. In women, BC alone is responsible for 15% of all cancer-related fatalities and 25% of all cancer incidences. Approximately 38% of BC deaths and half of all BC cases occur in developed nations. (4,20).

A statewide investigation in Denmark in 2016 discovered a lower risk of BC in hypothyroidism and an increased risk in hyperthyroidism (24). Similarly, Hyperthyroidism was associated with a significantly higher risk of BC mortality after 60 years of age in a prospective cohort study of US women, but not at younger ages. The multivariable-adjusted risk of breast cancer mortality was estimated to be about two-fold higher among women with hyperthyroidism compared to women without thyroid disease (25). According to another Brazilian study, thyroid disease affected 58% of BC patients, and 31% of them had subclinical hyperthyroidism (26). According to another Italian study, the prevalence of thyroid disorders was 14 in 100 (14%) in controls and 47 in 102 (46%) in BC patients (27).

A 2021 meta-analysis reported that hypothyroidism was linked to a slightly reduced BC risk, potentially due to decreased breast cell proliferation caused by lower thyroid hormone levels (28). However, a 2020 study found no significant association between hypothyroidism and BC risk but noted an increased risk in women with thyroiditis, emphasizing the need for closer monitoring (29).

Globally there are about one billion people with vitamin D deficiency (30) Vitamin D deficiency is a severe health issue with multiple health repercussions including BC (31). According to a previous systematic review using a different cut-off value within specific regions vitamin D deficiency affected 30-90% of people in developing countries (32). Three out of ten Africans had vitamin deficiency (less than 50 nmol/L 25(OH) D concentration), and one in five of the individuals had severe vitamin D deficiency (less than 30 nmol/L 25(OH) D concentration (33).

Low-level vitamin D exposure increases the risk of BC. Evidence indicates that vitamin D deficiency prevalence's in the general population with BC ranges from 23% (in Chinese) to 95.6% (in Pakistan) (34). Another study in Indonesia also reported that 82.4% of BC women have SVDD (35). More than 50% of newly diagnosed premenopausal women with BC had very

severe or severe vitamin D deficiency (36). The post-menopausal women who have 25 (HO)D deficiencies had also 7.5-fold greater risk of BC compared with the control group (37). Another study also reported serum levels of vitamin D ≥40.6 ng/mL could be considered protective against the risk of developing BC (38). A study from Ethiopia reported, 91.9% of chemotherapy-naive BC were vitamin D deficient with 41% being severely deficient (SVDD) (39).

BC is also a primary cause of mortality from cancer in low-income countries of the world. This is partly because a shift in lifestyles is causing an increase in incidence, and partly because clinical advances to combat the disease are not reaching women living in these regions (40). According to ministry of health 2022 report in Ethiopia, about 40,000 people die from BC every year (41).

While numerous studies worldwide have examined the associations between vitamin D, thyroid function, and BC, limited research has been conducted in Ethiopia. Therefore, this study aims to investigate the relationship between BC, serum vitamin D levels, and thyroid function tests among newly diagnosed female BC patients at Tikur Anbessa Specialized Hospital (TASH) in Addis Ababa, Ethiopia.

1.3 Significance of the Study

To the best of our knowledge, very limited studies were found on the status of thyroid function profiles and vitamin D status among breast cancer patients in Ethiopia. This study's main objective was to evaluate levels of serum vitamin D and thyroid function tests in BC patients at TASH. This study is expected to raise awareness about the need to employ serum vitamin D and thyroid function testing for determining a BC diagnosis. This study also can be used as a valuable resource for patients, clinicians, the community, and legislators interested in prevention and risk reduction. Furthermore, this study will provide baseline data for other scholars researching this topic.

To enhance the outcomes of the patients, this study concentrates on vitamin D and thyroid status in BC patients by giving information for accurate and fast diagnosis, medication, and monitoring. Due to their predictive or diagnostic applications, the identification of supportive tests can thus aid in lowering mortality rates.

2. Literature Review

2.1 Thyroid gland and the synthesis of thyroid hormone

The thyroid gland, a butterfly-shaped endocrine gland located in the front of the neck, secretes thyroxin (T4) and triiodothyronine (T3) through the action of thyroid peroxidase (TPO) and thyroglobulin. The thyroid gland is the only source of T4 and around 20% of T3; however, the remaining 80% of T3 is synthesized by the enzymatic elimination of T4's outer ring 5′-iodine. Thyrotropin (thyroid stimulating hormone, TSH), a glycoprotein hormone produced by the basophilic cells of the adenohypophysis, regulates thyroid gland function (18,42). T4 and T3 in circulation are primarily bound to thyroxine-binding globulin, transthyretin, and albumin, with only 1% remaining unbound and physiologically active. T4 is converted to T3 in peripheral tissues by type 1 and type 2 deiodinases; T3 hits thyroid receptors and initiates target gene expression (43).

Thyroxine, usually known as T4 because it contains four iodine atoms, is the most important thyroid hormone secreted by the thyroid gland. T4 is transformed to triiodothyronine (T3) by the removal of an iodine atom to exert its effects. This happens primarily in the liver and in areas where T3 activates, such as the brain. Thyroid stimulating hormone, which is produced in the pituitary gland at the base of the brain, regulates the quantity of T4 produced by the thyroid gland (TSH). The amount of TSH released into the bloodstream by the pituitary gland is determined by the amount of T4 present. If the pituitary gland detects a low level of T4, it creates more TSH to signal the thyroid gland to make more T4. When T4 levels in the blood are above a particular threshold, the pituitary gland's generation of TSH is inhibited (18,44).

2.2 The association of Breast Cancer and Thyroid Function Tests

The impact of thyroid axis dysfunctions on breast cancer growth has been a source of controversy for more than a century, and many questions remain. Thyroid hormones were used to treat BC patients as early as the nineteenth century. Thyroid Disorder may affect BC progression in several ways, including altered plasma levels of TSH and thyroid hormones or the production of specific thyroid autoantibodies, dysregulation of prolactin(PRL) secretion due to hypothyroidism, and changes in THs responsiveness of BC cells (45).

The prevalence of comparable pathophysiological pathways in both glands supports the possibility of a link between thyroid dysfunction and breast cancer. Thyroid peroxidase (TPO), myeloperoxidase, (MPO) and lacto peroxidase (LPO) are immunologically similar enzymes (33% amino acid similarity)found in thyroid and breast tissue (46). TH (T3 and T4) are shown to affect cancer growth, apoptosis, invasiveness, and angiogenesis, those hormones are also affect BC cells by a variety of non-genomic routes, including activation of the plasma membrane receptor integrin αVβ3 (a plasma membrane integrin that functions as a membrane receptor for TH). Furthermore, dysregulation of TH local bioavailability affects cancer development and progression. Other components of the TSH/thyroid hormone axis, such as TSH and selenoiodinase enzymes, have putative effects in breast cancer pathophysiology(16).

In conclusion, from all the information acquired so far, it appears that alterations of the hypothalamus–pituitary–thyroid axis could affect BC progression in several ways, as schematically depicted in Fig 1. In the next paragraphs, we will attempt to address each of these aspects in the light of the most recent epidemiological, clinical, and experimental findings.

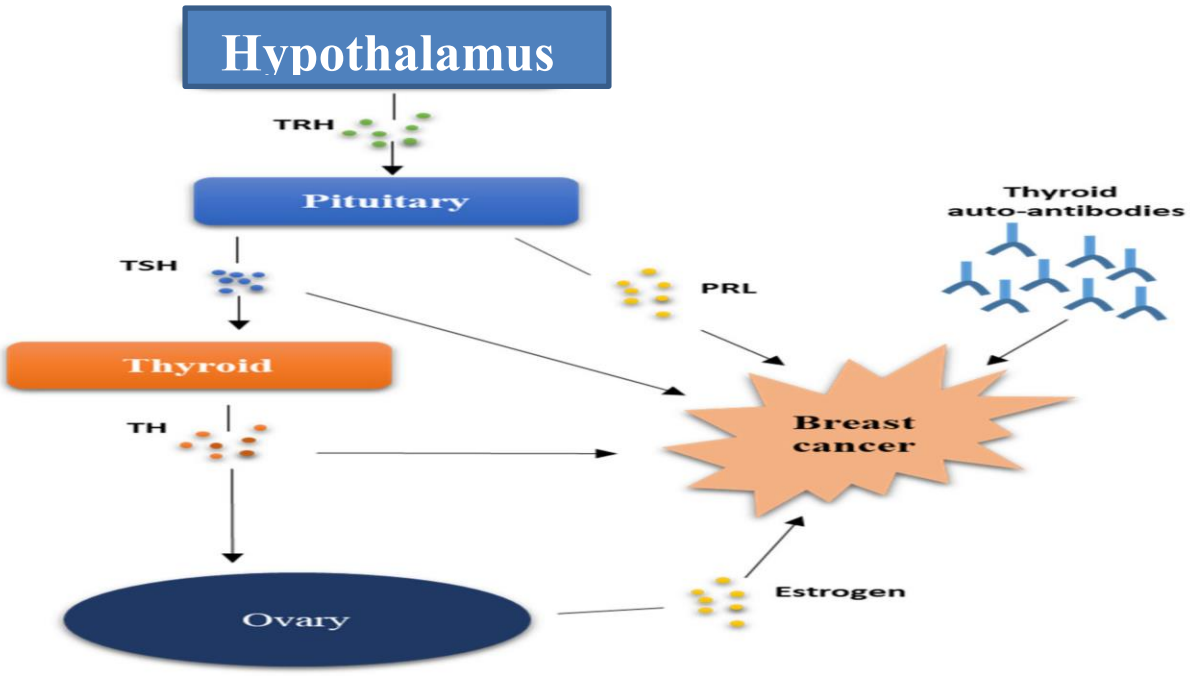

Figure1. Regulation of breast cancer progression by the hypothalamic–pituitary–thyroid (HPT) axis; involves Thyroid Releasing Hormone (TRH)-induced TSH secretion, modulating TH and PRL levels, which influence tumor proliferation, metabolism, and hormone responsiveness (45).

A case-control study conducted in three Mexican states (2004-2007) with 682 breast cancer (BC) patients and 731 controls found that higher serum Total Thyroxine (TT4) and lower Total Triiodothyronine (TT3) concentrations were linked to increased BC risk in both premenopausal and postmenopausal women. A strong positive association between BC and TT4, and a strong negative association with TT3 levels were observed (47).

In a case-control study of 143 BC patients and 128 healthy controls in Greek; the mean values of serum TH levels were 2 1.18mIU/ml for TSH, 9.7ng/dl for FT4 and 2.34ng/ml for FT3. In the control group, the comparable levels were 1.29ng/ml, 9.65ng/ml, and 1.6mIU/ml respectively. Thyroid hormone levels in BC patients and controls did not differ statistically significantly (48).

There was another case-control research carried out in 2005 between 26 Brazilian BC patients and 22 control groups to analyze the TH profile of BC patients; the findings revealed that Thyroid disease incidence was higher in patients than in controls (58 vs. 18%, $P < 0.05$). Subclinical hyperthyroidism was the most frequent disorder in BC patients (31%) and hypothyroidism (8%), hyperthyroidism was also associated with postmenopausal BC patients, as evidenced by significantly higher mean TT3 and TT4 values and lower TSH levels in this group of BC patients than in controls (26). In 2014, a comparative study conducted among 120 study participants in Saudi Arabia, demonstrated that TT3 and TT4 levels were substantially higher in BC patients than in healthy controls. It was concluded that hyperthyroidism had a significant impact on breast cancer cell growth (49).

In 2017, an Iranian case-control research was conducted with 86 BC patients and 50 controls. The mean blood TSH level did not differ substantially between the two groups; however patients had greater mean serum levels of T4 ($p<0.05$). TSH and TT4 levels were elevated in 5.8% and 2.3% of patients, respectively, and in 12% and 8% of the control group. TSH and T4 levels were also found to be lower in 7% and 2.3% of patients, respectively, and 5% and 0% of the control group.TT3 levels were elevated in 0% and 4% of patients and controls, respectively (50).

A prospective observational study also carried out among 86 Libyan women BC patients in 2018. No discernible variation was seen in TT4 and TT3 measurements among BC groups and healthy groups, but there was a significant difference in TSH measurements when comparing pre-treatment BC patients and the control group ($p=0.016$) (51).

This knowledge may highlight the biological link between thyroid disorders and breast cancer, making thyroid profile assessments in BC patients valuable for their clinical care.

2.3 Sources, Synthesis, and Metabolism of Vitamin D

Vitamin D is referred to as a steroid hormone and consequently is one of the most significant physiological regulators of calcium, phosphorus, and bone metabolism. Vitamin D exists in two chemical forms: ergocalciferol (vitamin D2), which plants produce, and cholecalciferol (vitamin D3), which is on produced in animal tissues and in human skin by the action of near-ultraviolet (290-310 nm) light 7-dehydrocholesterol. Both are physiologically inactive prohormones that must be 25-hydroxylated and 1-hydroxylated before they can bind to and activate the vitamin D receptor (52).

Vitamin D is first converted to 25(OH) D, the major circulating metabolite, by 25-hydroxylases in the liver. 25(OH) D then undergoes a second hydroxylation in the kidney into 1, 25 dihydroxyvitamin D (1, 25(OH) 2D), by 1- α -hydroxylase (CYP27B). 1,25(OH)2D, also known as calcitriol, is the biologically active form of vitamin D and exerts its action by binding to an intracellular receptor, the vitamin D receptor (VDR) (30).

2.4 The association of Breast Cancer and Vitamin-D

Vitamin D influences the natural history of cancer through multiple mechanisms. These include the role of vitamin D in apoptosis induction, cell differentiation stimulation, anti-inflammatory and anti-proliferative effects, and inhibition of angiogenesis, invasion, and metastasis (10). The several ways by which 1, 25-dihydroxy vitamin D (1,25(HO)2 D) can protect against cancer include apoptosis induction, cell differentiation promotion, anti-inflammatory and anti-proliferative actions, and suppression of angiogenesis, invasion, and metastasis (53).

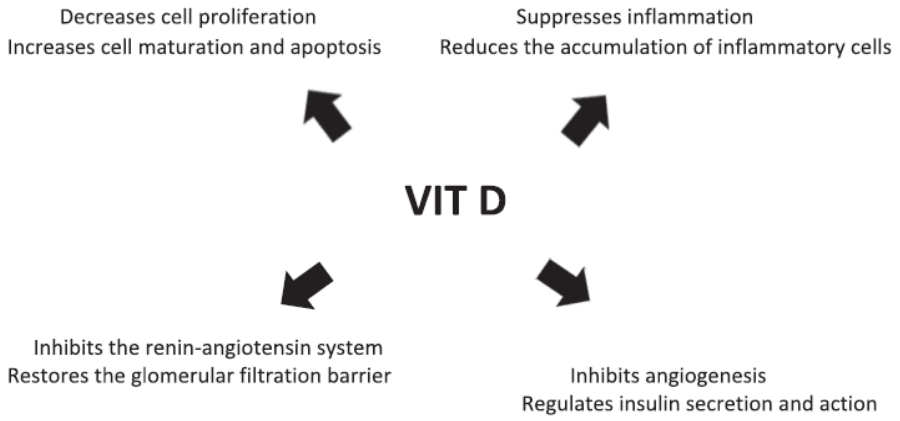

Figure 2: Summary of the mechanisms of action of vitamin D in the breast cancer (10).

A comparative, cross-sectional clinical investigation research was carried out in Brazil to assess the prevalence and risks associated with low pretreatment 25(HO) D levels in postmenopausal BC women compared to postmenopausal women without BC. The rates of insufficient (20-29ng/mL) and deficient (<20ng/mL) 25(OH)-D levels were higher than in controls (55.6% vs. 49.3%, P=0.039 and 26.2% vs. 20.3%, P=0.018). BC patients had a 1.5-fold higher chance of acquiring low Vitamin-D levels (OR=1.52, 95% CI, 1.04-2.22, P=0.029) than women without BC in a risk analysis (adjusted for age, time since menopause, and BMI). At the time of BC diagnosis, postmenopausal women were at an increased risk of vitamin-D deficiency (54).

A case-control research conducted in Pakistan titled vitamin D deficiency in newly diagnosed BC Patients from November 2010 to May 2011. BC patients had considerably lower serum vitamin D levels (9.3ng/mL) than the control group (14.9ng/mL). Vitamin D deficiency (less than 20 ng/mL) was observed in 95.6% of cases and 77% of the control group (P <0.001), whereas Vitamin D insufficiency (20-39 ng/mL) was observed in only 4.4% of patients and 18.9% of healthy females. However, contrary to tumor characteristics (histology grade, stage, and receptor status) did not show any significant associations with serum vitamin D levels. The mean serum vitamin D levels in women with premenopausal and postmenopausal BC were 10.5 and 13.5ng/ml, respectively (P value 0.015) (55).

Another case-control study in the same area, involving 94 study participants, discovered that serum vitamin D levels in patients were significantly lower than in controls. Vitamin D levels in 85.7% of cases and 55.8% of control groups were less than 20ng/ml. Only 14.3% of breast cancer patients had vitamin D levels more than 20ng/ml (56).

In Iran, a cross-sectional study was done between 2013 and 2017 to examine the association between serum vitamin D levels and prognostic variables in 214 women with BC who did not have metastatic disease. The serum 25 (HO) D concentration was 25.15 ± 17.68ng/ml. Vitamin D levels had no significant link with disease stage, tumor size, cancer grade, human epidermal growth factor receptor 2, progesterone receptor, or estrogen receptor (57).

From June 2013 to June 2017, a prospective study was done in 50 Egyptian women with BC to look at the frequency and prognostic significance of inadequate vitamin D. Vitamin D insufficiency was seen in 15 (30%) of the patients and was associated with larger tumor size ($p = 0.001$), higher grade ($p = 0.014$), advanced stage ($p = 0.001$), lymph node positivity ($p = 0.012$), and HER2/neu expression ($p = 0.002$) (23). Although low serum vitamin D levels were detected in BC patients in another case-control research conducted among 114 study participants in Egypt, the relationships with larger tumors, lymph node involvement, advanced stage, and metastatic disease in the study were not statistically significant (58).

This finding may clarify the link between vitamin D levels and breast cancer, suggesting that assessing serum vitamin D in BC patients could provide valuable insights for their management.

3. Objective

3. 1 General Objective

- To assess serum vitamin D and thyroid function test levels among newly diagnosed female BC patients attended in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

3.2 Specific Objectives

- To assess Serum thyroid profiles in breast cancer patients and healthy control groups
- To assess Serum 25(HO)D levels in breast cancer patients and healthy control groups
- To compare 25(OH) D levels and thyroid function across different breast cancer stages
- To assess the factors associated with thyroid function tests and vitamin D levels

4. Materials and methods

4.1 Study area

This study was conducted in the adult oncology center of TASH, located in Ethiopia's capital city of Addis Ababa (9^{o}N, 38^{o}E, and 2440 meters above sea level height). TASH was established in 1972. It is Addis Ababa University's affiliated with the College of Health Sciences, the major teaching public hospital. For undergraduate and graduate students studying medicine, pharmacy, and other health sciences, it serves as a training facility. There are approximately 992 nurses, 115 other healthcare professionals, 76 pharmacists, and 465 doctors working at the hospital. It also employs 950 support and administrative personnel. With about 700 beds, the hospital treats over 500,000 patients annually through its emergency rooms, inpatient clinics, and 20 outpatient specialty clinics. The adult oncology center treats around 850 patients per month and over 10,000 patients per year (39,59). This hospital was purposively selected to recruit study participants because it is among the biggest referrals to chemotherapeutic service centers in the country.

4.2 Study Design and Period

A facility-based comparative cross-sectional study was carried out at Tikur Anbessa Specialized Hospital (TASH) from January to March 2024.

4.3 Population

4.3.1 Source of Population

- All female patients with BC who were attending TASH served as the population's source.
- The Source population for the control groups was all healthy female individuals.

4.3.2 Study population

- All volunteer new BC patients who were admitted and met the requirements for inclusion during the study period.
- All healthy volunteer female individuals who fulfilled the criterion for inclusion.

4.4 Inclusion and Exclusion Criteria

4.4.1 Inclusion criteria

- The study included females newly diagnosed with breast cancer (via mammography), regardless of stages and menopausal status.
- The control group included apparently healthy with no family history of BC females

4.4.2 Exclusion criteria

- Under treatment for BC under radiation and hormonal or chemotherapy
- Taking vitamin D supplements
- Pregnancy and lactate for the last 6 months
- Patients with self-reported thyroid problems or currently taking thyroid medications
- Diabetic patients
- Clients extremely ill and unable to provide sample and consent

4.5 Study Variables

4.5.1 Dependent Variables

- Thyroid profiles (TSH, TT3, TT4, FT3 and FT4) level
- Serum Vitamin D level

4.5.2 Independent Variables

- Social and demographic aspects (ages, Educating attainment, marital and occupational statuses, and)
- Body Mass Index (BMI)
- Stages of breast cancer
- Serum total calcium (Ca) and phosphate (Phos)
- Pre-menopausal and post-menopausal status
- Exposed to Sunlight
- Serum Reproductive tests (E2, PRL and Prog.)
- Types of Breast cancer(Invasive ductal, Invasive lobular, Noninvasive ductal)
- Behavioral characteristics (Alcohol consumption, Khat chewing, smoking habits)
- Use of Contraceptive
- Parity number

4.6 Sample size determination and sampling technique

4.6.1 Sample size determination

We calculated the sample size using two population means formula for all specific objectives. We assumed the true population mean (variance) of FT4 in cases 1.43 (0.63) ng/dL, while in control 1.10 (0.23) ng/dL; 95% confidence level, 80% estimation power, and ratio case to control 1:1. We also assumed equal variance. After we substitute the above assumed numbers in the formula below:

$$n_1 = n_2 = \frac{(Z_{1-\alpha/2} + Z_\beta)^2 * (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

Where, the n_1-desired sample size for cases, n_2-sample size for control, mean of FT4 in cases = 1.43, mean of FT4 in control =1.10, $\sigma_1^2 = 0.63 \; and \; \sigma_2^2 = 0.23$ variance for cases and control respectively based on a previous study in Brazil post-menopause breast cancer women (26), $Z_{1-\alpha/2}$- critical value under a standard normal distribution curve for a 95%CI ($Z_{1-\alpha/2} = 1.96$) and Z_β- critical value under a standard normal distribution curve for 80% estimation power ($Z_\beta = 0.84$).

$$n_1 = n_2 = \frac{(1.96 + 0.84)^2 * (0.63 + 0.23)}{(1.43 - 1.0)^2}$$

$n_1 = n_2 = 62$. However, the total sample size for the two groups is equal to 124.

Since the sample size calculated for the first objective was the highest, we used this sample size as this study's sample size. The sample size was increased by assuming a 10% non-response rate and errors during sample collection and test. Accordingly, 138 participants from both groups were enrolled in this study. According to the calculated sample size, a total of was 62 but it was set to 69 by adding a 10% non-response rate. Therefore, 69 samples were taken from each group, resulting in a total sample size of 138.

4.6.2 Sampling Technique

Convenient sampling was employed in order to decide cases and controls based on the inclusion and exclusion criteria. Convenience sampling was used due to practical constraints, such as time limitations and the availability of newly diagnosed BC patients at the study site.

4.7 Measurement and Data Collection

4.7.1 Method of data collection and Tools

Depending on the study objectives, different data collection tools were used to gather pertinent information. Patient data was collected through face-to-face interviews conducted by trained data collectors, document reviews, and pathology results. The following characteristics were obtained using a structured questionnaire: age, marital status, parity, and educational and occupational status. Body weight and height were measured by trained nurses using a digital weighing scale with a height rod, and BMI was calculated as weight (kg) divided by height squared (m^2) (BMI = kg/m^2). Blood specimens were collected by laboratory personnel following standard procedures. Clinical characteristics, including the type and stage of breast cancer, were extracted from patient medical records by the research team using a structured data collection format.

4.7.2 Collection of Samples

Blood specimens were collected after written informed consent was obtained from each participant. A blood sample of approximately 3–5 milliliters was taken from each participant, in Serum Separator Tube (SST) following a Standard Operating Procedures (SOP). The sample was collected by trained nurses, using a sterile disposable syringe and an aseptic standard non-traumatic vein puncture technique.

Following collection, the samples were centrifuged at 4000 revolution per minute **(**rpm**)** for five minutes. The resulting serum was transferred into 1.5 mL Eppendorf tubes, labeled with the same participant identification code as the SST, and transported to the EPHI using a triple packaging system. After that, the sample was kept at EPHI at -80°C deep freezer until it was analyzed.

4.7.3 Laboratory Analysis and Tests Principle

The samples were examined for TSH (in µU/mL), FT3 (in pg/mL), FT4 (in ng/dL), TT4 (in µg/dL), TT3 (in ng/mL), Prog, PRL, E2, Ca (in mmol/L), Phos (in mmol/L) and 25(HO) D (in ng/ml) concentrations using the COBAS 6000 at the Clinical Chemistry Lab. of EPHI, that is nationally recognized by the EAS. The COBAS 6000 is a fully automated machine produced by Roche Diagnostics GmbH, Mannheim, Germany. This device uses the Electrochemiluminesc ent Immunoassay (ECLIA) principle using the e601 part to measure the levels of TSH, FT3,

FT4, TT3, and TT4 and 25(HO) D and spectrophotometer using the c501 part to measure serum Ca, Phos concentrations

Test principle for serum FT3, FT4, TT4, TT3, and 25(HO) D Concentrations

The competitive principle is applied to analytes of low molecular weight, such as FT3, FT4, and 25(HO) D.

- ✓ In the first step, sample and a specific anti-T3/T4 antibody labeled with a ruthenium complex are combined in an assay cup.
- ✓ After the first incubation, biotinylated T3/T4 and streptavidin coated paramagneticmicrobead s are added. The still free binding sites of the labeled antibody become occupied, with formation of an antibody-hapten complex. The entire complex is bound to the micro-bead via interaction of biotin and streptavidin.
- ✓ After the second incubation, the reaction mixture containing the immune complexes is transported into the measuring cell. The immune complexes are magnetically entrapped on the working electrode, but unbound reagent and sample are washed away by Pro Cell
- ✓ In the ECL reaction, the conjugate is a ruthenium based derivative and the chemiluminescent reaction is electrically stimulated to produce light. The amount of light produced is indirectly proportional to the amount of antigen in the patient sample.

Evaluation and calculation of concentration of the antigen are carried out by means of a calibration curve that is established using standards of known antigen concentration.

Test principle for serum TSH level

The sandwich principle is applied for thyroid-stimulating hormone (TSH).

- ✓ In the first step, patient sample is combined with a reagent containing biotinylated TSH antibody and a ruthenium-labeled TSH-specific antibody in an assay cup. During a 9-minute incubation step, antibodies capture the TSH present in the sample.
- ✓ In the second step, streptavidin-coated paramagnetic micro-beads are added. During a second 9-minute incubation, the biotinylated antibody attaches to the streptavidin-coated surface of the micro beads

- ✓ After the second incubation, the reaction mixture containing the immune complexes is transported into the measuring cell; the immune complexes are magnetically entrapped on the working electrode, but unbound reagent and sample are washed away by Pro-cell.

In the ECL reaction, the conjugate is a ruthenium based derivative and the chemiluminescent reaction is electrically stimulated to produce light. The amount of light produced is directly proportional to the amount of TSH in the sample. Evaluation and calculation of concentration of the antigen or analyte are carried out by means of a calibration curve that was established using standards of known antigen concentration(60) **(Annex VI).**

4.8 Data Quality Assurance

Training sessions were provided to data collectors, including nurses and laboratory personnel, covering ethical considerations, interviewing techniques, proper handling of clinical data, and standard procedures for blood sample collection. The training also included practical demonstrations and role-playing exercises to ensure consistency. To maintain data quality, the primary investigator and data collectors cross-checked each completed questionnaire for completeness. Clinical data were extracted using a structured format, and questionnaire data and lab results were double-entered into a pre-prepared Excel database for quality assurance.

4.8.1 Pre-analytical

The quality of blood samples was ensured by adhering to standard operating procedures during collection, processing, and transportation. Samples were checked for hemolysis, clotting, volume, collection time, and proper labeling before analysis. They were transported to National Clinical Chemistry Reference Laboratory (NCCRL), EPHI in a temperature-controlled container to maintain stability and prevent degradation. The manufacturer's procedures, safety precautions, and specimen handling protocols were strictly followed throughout the process.

4.8.2 Analytical

Analytical quality assurance was conducted by well-trained and experienced laboratory professionals at the National Clinical Chemistry Reference Laboratory (NCCRL) of EPHI. For the corresponding measured parameters, standard operating procedures (SOPs) were closely adhered to. Before proceeding with the tests, the performance of the COBAS 6000 was verified

by running normal and pathological quality control samples, and the outcomes were assessed using the manufacturer's control data.

4.8.3. Post-analytical

The results of each vitamin D and thyroid function tests were printed out following a review to ensure all test results were proper and complete. The data was carefully entered directly into a SPSS software data sheet and was set aside for statistical examination.

4.9 Data Analysis and Interpretation

SPSS version 20 was used for data entry. The chi-squared test analyzed categorical variables such as marital status, parity, education, occupation, menopausal status, contraceptive use, smoking, and Khat chewing. The Kolmogorov-Smirnov test assessed normality in continuous variables. Parametric tests, including the independent t-test and one-way ANOVA, were used for normally distributed variables (TT4, FT4, and TT3). For non-normally distributed variables (TSH, FT3, E2, PRL, Prog, 25(OH)D, Ca, and Phos), which remained skewed despite log or square root transformation, the Mann-Whitney U and Kruskal-Wallis tests were applied. Multiple linear regressions used to assess associated factors for thyroid function test and 25(HO) D levels, with statistical significance set at $P < 0.05$.

4.10 Operational definitions

- **Cases:** Newly diagnosed female breast cancer-positive patients (pathology proven) who weren't in any treatment service at Tikur Anbessa Specialized Hospital.
- **Control Group**: females with no history of BC familly who presented to OPD for compl ain other than breast pathology (with normal liver function and renal function tests)
- **thyroid Function profiles:** TSH, TT3, TT4, FT3 and FT4
- **25(HO) D:** used to help in the measurement of vitamin D status and contains both forms of vitamin D; 25(HO) D3 and 25(HO) D2.
- **25(HO) D:** used as an aid in the assessment of vitamin D status and contains both forms of vitamin D; 25(HO) D3 and 25(HO) D2.

Vitamin Status: Accordingly, severe vitamin D deficiency (SVDD) and vitamin D deficiency (VDD) were defined as circulating 25(OH) D levels of < 10 ng/L, and 10–20 ng/L, respectively, whereas levels above 20 ng/mL were considered sufficient (normal) (73).

Thyroid dysfunction Classification:

- The expected normal values for TT3 and TT4 were (0.8-2.0 ng/mL) and (4.5-11.7 µg/dL) respectively.
- Subclinical hypothyroidism: Elevated TSH beyond the reference range (0.4-4.2µIU/mL) with FT4 (0.93-1.71 mg/dL) and FT3 (2.02-4.43 pg/mL) levels within the normal range.
- Clinical hypothyroidism: Elevated TSH beyond the reference range with low FT4 and FT3 levels.
- Subclinical Hyperthyroidism: TSH levels <0.4 µIU/mL with normal FT4 and FT3 levels.
- Clinical hyperthyroidism: Low TSH levels with FT4 and FT3 above the reference range based on previously published article (61).

4.11 Ethical considerations

Ethical approval was received from the Department of Medical Laboratory Science at Addis Ababa University College of Health Sciences (approval number of DRERC/744/24/MLS). Permission was obtained from Tikur Anbessa Hospital's research ethics committees. All collected data were analyzed anonymously to ensure patient privacy. Prior to the start of data collection, each participant provided written informed consent. Access to softcopy and hardcopy of data was limited by password and key lock respectively to ensure confidentiality.

4.12 Dissemination of the result

Upon completion of the research, the finding of the study was submitted to Department of Medical Laboratory Science, Addis Ababa University and Ethiopian Public Health Institute. Oral presentation of the thesis will be made for both institutions. In addition the finding will also be presented on annual conferences. Up on completion of thesis defense, the findings of the study will be published on one of the peer reviewed journals.

5. Results

5.1. General characteristics of the study participants

The study included 138 female participants, 69 females who were newly diagnosed with BC(cases), and 69 healthy females (controls). The mean age of cases was 45.3 (±12) years, while 45.8 (±11.6) years for controls. The majority of cases 47 (68.1%) and controls 51 (73.9%) were under the age of 50 years (Table 1). The majority (59.4%) of females from the cases and controls (44.9%) were post-menopause. The average Body Mass Index (BMI) of the cases was 23.4 (±3.5) Kg/m2 and 22.9 (±1.8) Kg/m2 controls which was within the normal range. However, 8(11.6%) of cases were underweight, while 20 (29%) overweight. Moreover, 16 (23.2%) of cases and 12 (17.4%) of controls were consumed alcohol. Almost twice as many cases smoked cigarettes compared to the controls (14.5% versus 5.8%). Stages and types of BC by reviewing of patient card showed most patients (36.2%) were at stage 2 and By invasiveness, 58% had invasive ductal carcinoma (Table 1).

Table. 1: Sociodemographic and Clinical characterstics of the participants at TASH, Addis .Ababa, Ethiopia, 2024

Variables	**Classifications**	**Cases (n=69)**	**Controls (n=69)**	P-Value
		Frequency (%)	**Frequency (%)**	
Age group	≤ 50	47 (68.1)	51 (73.9)	0.470
	>50	22 (31.9)	18 (26.1)	
Marital status	Single	15 (21.7)	13 (18.8)	0.672
	Married	54 (78.3)	56 (81.2)	
Educational status	No formal education	29 (42)	26 (37.7)	
	Secondary or less	23 (33.3)	35 (50.7)	0.053
	Diploma and above	17 (24.6)	8 (11.6)	
Occupation	Unemployed	6 (8.7)	9 (13)	0.412
	Employed	63 (91.3)	60 (87)	
BMI	Underweight-Normal(<25 kg/m2)	47 (68.1)	62 (90)	**0.002**
	Overweight-Obese(>25kg/m2)	22 (31.9)	7 (10.1)	
Parity Number	None(0)	9 (13)	10 (14.5)	
	1–3	37 (53.6)	32 (46.4)	0.236
	≥4	23 (33.3)	27 (39.1)	
Menopausal Status	Pre menopause	28 (40.6)	38 (55.1)	0.088
	Post menopause	41 (59.4)	31 (44.9)	

Smoking condition	Yes	10 (14.5)	4 (5.8)	0.091
	No	59 (85.5)	65 (94.2)	
Contraceptive	Yes	32 (46.4)	22 (31.9)	0.485
	No	37 (53.6)	47 (68.1)	
Family history of BC	Yes	19 (27.5)		
	No	50 (72.5)		
Stage of BC	Stage I	11 (15.9)		
	Stage II	25 (36.2)		
	Stage III	19 (27.5)		
	Stage IV	14 (20.3)		
Types of BC	Non-invasive ductal	10 (14.5)		
	Invasive ductal	40 (58)		
	Invasive Lobular Carcinoma	19 (27.5)		

Variables were expressed in frequencies (f) and percentages (%) of the total (69 casese and 69 controls). p-value were calculated using pearson Chi-Square tests.

5.3 Levels of 25(OH) D and Thyroid Function Tests among the stages of BC patients

Thyroid function and vitamin D levels vary significantly across breast cancer stages. TT4 and FT4 levels are notably lower in Stages II and IV, while FT3 and 25(OH) D levels show a significant decline in advanced stages. However TSH and TT3 were not statistically significant at all of the stages (Table 2).

Table 2: Biochemical parameters among the four stages of pathologically confirmed BC patients (n=69) in TASH, Addis Ababa, Ethiopia, 2024.

Parameters	**Stage I (n=11)**	**Stage II (n=25)**	**Stage III (n=19)**	**Stage IV (n=14)**	**p-value**
TT4 (Mean±SD)	7.4±1.73	6.7±1.77[a]	8.9±2.7[ab]	6.6±1.9[b]	P<0.05[ab]
FT4 (Mean±SD)	1.1±0.14	1.03±0.18[a]	1.3±0.23[ab]	1.02±0.28[b]	P<0.05[ab]
TT3 (Mean±SD)	1.3±0.19	1.2±0.19	1.3±0.19	1.3±0.48	0.303
TSH Median(IQR)	2.0(1.6–2.4)	1.2(0.54–2.7)	1.5(1.2–2.1)	1.7(1.1–3.2)	0.515
FT3 Median(IQR)	2.9(2.8–3.5)[a]	2.6(2.3–3.0)	2.7 (2.5–3.1)	2.1(1.3–3.0)[a]	<0.05[a]
25(HO)D Median(IQR)	16.2(14.5-22.5)[c]	12.1(9–15.3)	15.3(10.4–19.1)	9.5(6.5–16.1)[c]	<0.05[c]

One-way ANOVA tests for mean ± SD (standard deviation) were used for the normally distributed data, however, the Kruskal Wallis Test for Interquartile Range (IQR) was used for

the not normally distributed data, <0.05=significant, The letters denote significant differences between groups as follows: a(TT4)=significance between Stage two and three, b(TT4)= significance between Stage three and four; a(FT4)=significance between Stage two and three, b(FT4)=significance between Stage three and stage four; a(FT3)=significance between Stage one and four, a(25(OH) D) significance between stage one and four.

5.3 Comparison of serum biochemical parameters among cases and Control groups

Figure 1 depicts the mean concentration of TT4, FT4, and TT3 between case and control groups. The results of an independent T-test showed no noticeable difference in TT4 levels between the cases (mean±SD =7.4±2.2 µg/dL) and controls (mean±SD=7.7±1.5 µg/dL) (p= 0.941) (Fig 1). Similarly, no significant mean difference existed between the cases. (mean ± SD =1.1± 0.24 ng/dL) and controls (mean±SD=1.2±0.17 ng/dL) on the concentration of FT4 (p=0.138). However, there was a significant mean difference in serum TT3 concentration between the case group (mean±SD=1.2±0.28 ng/mL) and the control group (mean±SD=1.4 ±0.19 ng/mL) (p<0.001) (Fig. 3).

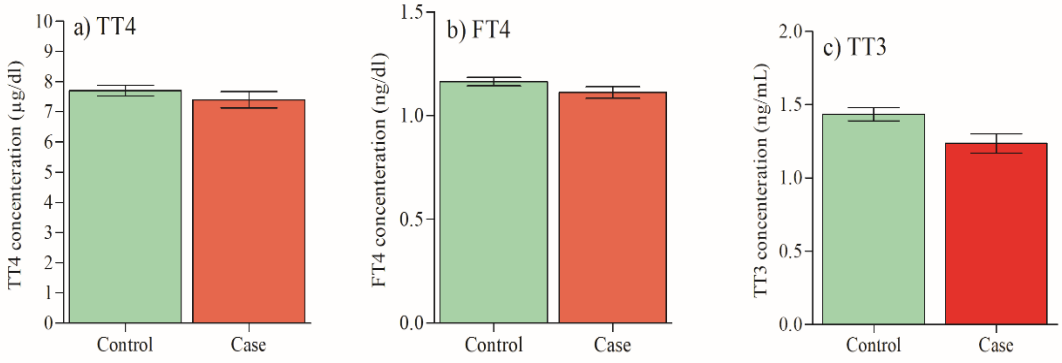

Figure 3: Mean comparison of a) TT4 in µg/dL, b) FT4 in ng/dL and c) TT3 ng/mL concentration between cases and controls.

The test of Mann-Whitney U revealed no notable variation in the median of TSH concentration between the cases (median=1.6 µU/mL, IQR=1.0–2.4 µU/mL) and controls (median=1.7 µU/mL, IQR =1.2–2.9 µU/mL) (P/0.416). In contrast, there was a significant difference in the median FT3 concentration between the breast cancer patients (median = 2.7 pg/mL, IQR =2.3–3.1 pg/mL) and controls (median =3.0 pg/mL, IQR =2.8–3.3 pg/mL) (P < 0.001) (Fig. 4)

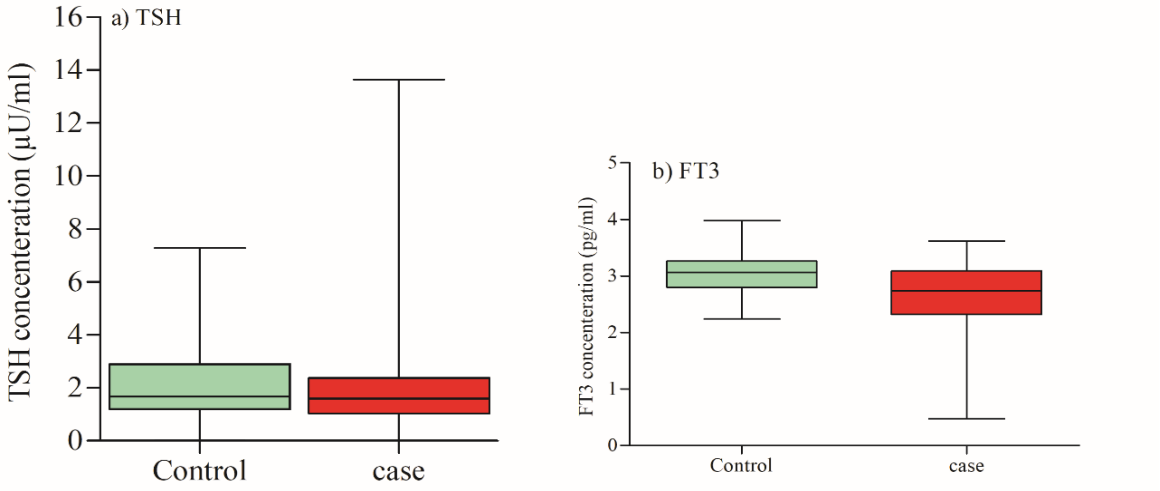

Figure 4: Median comparison between cases and controls on the concentrations of a) TSH in µU/ml and b) FT3 in pg/mL.

The Mann-Whitney U test revealed a significant higher in the median concentrations of serum E2, Prog, and PRL in the BC patients compared to the control group (Table 3).

Table 3: Comparison of serum reproductive tests in case and control groups in TASH, Addis Ababa, Ethiopia, 2024

Parameters	Cases (n=69) Median (IQR)	Controls (n=69) Median (IQR)	p-value
E2 (pg/mL)	11.7 (5 – 40.7)	5.0 (5.0 –5.0)	**<0.001**
Prog (ng/mL)	0.15 (0.09 –0.33)	0.05 (0.05–0.08)	**<0.001**
PRL (ng/mL)	10.3 (5.7 –17.6)	6.8 (5.7 –10.9)	**0.024**

P-value was assed using Man-Whitney test; E2 (Estradiol), Progesterone (Prog), Prolactin (PRL)

Figure 5 shows the median concentration of 25(HO) D, total calcium (Ca), and phosphate (Phos) among cases and controls. The Mann-Whitney U test showed that there was a statistically significant median difference in 25(HO) D concentrations between cases (median=14.5 ng/mL, IQR=9.9–15.8 ng/mL) and controls (median = 14.8 ng/mL, IQR = 12.7–19.7 ng/mL) (*p=0.043*). However, there was no discernible median difference in serum Ca between cases (median=2.3 mmol/L, IQR=2.1–2.4 mmol/L) and controls (median=2.3 mmol/L, IQR=2.2–2.4 mmol/L) (p=0.267). There was also an absence of a significant median difference in serum Phos. Levels between the cases (median=1.1 mmol/L, IQR=0.94–1.3 mmol/L) and controls (median=1.1 mmol/L, IQR=1.0–1.2 mmol/L) (*p= 0.162*).

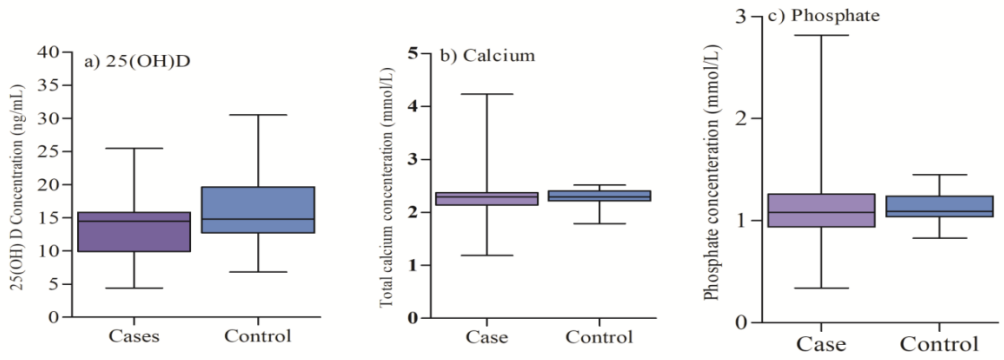

Figure 5: Median comparison between cases and controls on the concetration of a) serum 25-hy droxyvitamin D (25(HO)D) b)total calcium concentration (Ca), and c) serum Phosphate (Phos.).

5.5 Distribution of Thyroid Hormone Dysfunction and Vitamin D Status in the case and Control groups.

Table 4 shows that 7(10.1%) healthy control groups had some form of thyroid hormone pathology of which 4(5.8%) had subclinical hypothyroidism, and 3(4.3%) had subclinical hyperthyroidism. While from the cases, 11(15.9%) exhibited some form of thyroid hormone dysfunction; 5(7.2%) subclinical hyperthyroidism, 5(7.2%) subclinical hypothyroidism and 1(1.4%) clinical hypothyroidism. No clinical hyperthyroidism was observed in either group. Regarding the vitamin D status the majority (85.5%) of the cases involved developing vitamin D deficiency. However, a considerable proportion of the participants (26.1%) developed severe vitamin D deficiency (SVDD) in the cases. However, 72.4% of controls developed vitamin D deficiency, while 7.2% had SVDD. The severity of vitamin D deficiency had a significant association with BC (p=0.006)

Table 4: Distributions of Thyroid-Disorder and Vitamin D statuses in the case and control groups inTASH, Addis Ababa, Ethiopia, 2024

Classifications		**Cases (n=69)**	**Controls (n=69)**	
		Frequency (%)	**Frequency (%)**	**P-value**
Thyroid dysfunction		11(15.9)	7(10.1)	0.627
No thyroid dysfunction		58 (84.1)	62(89.9)	
	SVDD (<10ng/mL)	18 (26.1)	5 (7.2)	
Vitamin D	VDD (10-20ng/mL)	41 (59.4)	45 (65.2)	**0 .006**
Status	Sufficient (>20 ng/mL)	10 (14.5)	19 (27.5)	

p-value calculated using Pearson Chi-Square test.

5.6 Factors associated with thyroid function tests and vitamin D levels

Advanced breast cancer (Stage IV) was associated with lower 25(OH) D (ß = -1.9, 95% CI: -3.0, -0.7; p = 0.002), FT3 (ß = -0.4, 95% CI: -0.3, -0.1; p < 0.001), and TT4 (ß = -0.04, 95% CI: -0.07, -0.01; p < 0.001). Stage II showed reduced FT3 and TT4 levels, while Stage III exhibited elevated TT3 and FT4 levels. Invasive breast cancer subtypes, including Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC), were associated with decreased 25(OH) D levels and all thyroid function tests. Additionally, contraceptive use was linked to lower TT3 and FT4 levels, while progesterone levels positively correlated with FT4 (Table 5).

Table 5: Association of independent variables with the levels of 25(OH) D and Thyroid Function Tests in BC Patients and Controls in TASH, Addis Ababa, Ethiopia, 2024.

	Variables	**n(%)**	**25(HO)D (ng/mL)**	**TT3 (ng/mL)**	**FT3 (pg/mL)**	**TT4 (µg/dL)**	**FT4 (ng/dL)**	**TSH (µU/mL)**
			ß (95%CI);p-value	**ß(95%CI));p-value**	**ß(95% CI) ; p-value**	**ß(95%CI);p-value**	**ß(95%CI);p-value**	**ß(95% CI);p-value**
Age	<50 Years	92(66.7)	0	0	0	0	0	0
	≥50 Years	46(33.3)	-0.2(-6, 0.5);0.094	0.1(-0.06, 0.2); 0.366	0.1(-0.2,0.3); 0.597	0.05(-0.7, 1); 0.659	-0.1(-0.1, 0.1); 0.582	-0.04(-1.1, 0.8); 0.769
BMI	<25 Kg/m^2	109 (79)	0	0	0	0	0	0
	>25 Kg/m^2	29 (21)	-1(-4.8, 1.6);0.328	-0.1(-0.2, 0.04);0.225	-0.01(-0.2, 0.2);0.892	(-0.84, 0.84); 0.999	0.05(-0.1, 0.2);0.417	0.03(-0.7, 0.99);0.772
Contraceptive	No	84 (60.9)	0	0	0	0	0	0
	Yes	54 (39.1)	1.5(-1, 4); 0.226	-0.7(-1.4, 0.05);**0.036**	-0.(-0.2,0.2); 0.889	-0.1(-0.2,-0.1); **0.043**	0.6(-0.1, 1.2); 0.106	0.01 (-0.2, 0.2); 0.889
Smoking	No	124(89.9)	0	0	0	0	0	0
	Yes	14(10.1)	-2.6(-7, 1.6);0.216	-0.3 (-1.4, 0.8); 0.632	0.02(-0.3, 0.3); 0.793	-0.01 (-0.1, 0.1); 0.837	-0.6 (-1.7, 0.5);0.302	0.04 (-0.3, 0.3); 0.793
Menopause	Pre-menopause	66(47.8)	0	0	0	0	0	0
	Post-menopause	72 (52.2)	1.9 (-2.4, 6); 0.384	-0.66(-1.8,0.5); 0.246	-0.14(-0.5, 0.1);0.285	0.01 (-0.1, 0.1); 0.861	-0.74 (-2, 0.4); 0.194	-0.1(-0.4, 0.3); 0.648
	Controls	69(50)	0	0	0	0	0	0
Stage of BC	Stage I	11(8)	2.4(-2.9,8); 0.376	0.15(-1, 1.5); 0.829	-0.01(-0.4, 0.4);0.940	-0.1(-0.4,0.02); 0.099	0.7(-0.7, 2.1); 0.321	0.14(-0.3, 0.6); 0.519

	Stage II	25(18.1)	-2(-4, 0.5); 0.123	-0.15(-0.7, 0.4);0.602	-0.2(-0.3,-0.02);**0.026**	-0.1(-0.1, 0.01); **0.023**	-0.2(-0.8,0.3); 0.425	-0.1(-0.3, 0.14); 0.465
	Stage III	19(13.8)	0.1(-1.3,1.4);0.943	0.6(0.2, 0.9);**0.001**	0.1(-0.2, 0.02); 0.138	0.05(0.01, 0.1); **0.014**	-0.1(-0.4, 0.3); 0.769	-0.1(-0.4, 0.2); 0.462
	Stage IV	14(10.1)	-2 (-3,-0.7); **0.002**	-0.2(-0.5, 0.2); 0.296	0.4(-0.3,-0.1); **<0.001**	-0.04(-0.1,-0.1); **0.040**	0.2(-0.13, 0.5);0.248	0.02(-0.24, 0.3);0.898
	Controls	69(50)	0	0	0	0	0	0
Type of BC	Invasive Ductal BC	40(29)	-0.3(-4.6,-1);**0.003**	-0.4(-0.4, -0.1); **0.007**	-0.3(-0.7,-0.1); **0.019**	-0.1(-1.5, 0.8); 0.532	-0.3(-0.3,-0.1); **0.035**	-0.14(-1.8, 0.6); 0.334
	Invasive Lobular BC	19(13.8)	-0.2(-4, 0.1);0.063	-0.3(-0.2, -0.1); **0.002**	-0.2(-0.3,-0.04);**0.015**	-0.19(-1.1, 0.03);0.066	-0.2(-0.1,-0.1); **0.020**	-0.2(-1.1, -0.01);**0.045**
	No Invasive Ductal BC	10(7.2)	-0.2(-8, 1.1);0.137	-0.3(-0.2, 0.04);0.062	-1.4(-0.2, 0.04);0.176	-0.02(-0.9, 0.05);0.077	-0.1(-0.1,0.02);0.213	0.05(-0.6, 0.4); 0.676
	E2 (pg/mL)		0.1(-0.1, 0.1); 0.256	-0.01(-0.1, 0.1);0.519	0.04(0.0, 0.01); 0.636	(-0.01, 0.01); 0.636	(-0.01, 0.01); 0.636	-0.01(-0.01,0.1);0.286
	Prog (ng/mL)		0.1(-0.4,0.6);0.592	-0.05(-0.2, 0.1);0.444	-0.04(-0.2, 0.1);0.619	-0.1(-0.02, 0.01);0.223	0.2(0.03, 0.3); **0.018**	0.04(-0.02, 0.1);0.174
	PRL(ng/mL)		-0.1(-0.1,0.1); 0.557	-0.01(-0.1,0.1); 0.179	-0.2(-0.05, 0.0);0.085	-0.01(-0.1,0.01); 0.460	-0.01(-0.1,0.1);0.434	-0.01(-0.3, 0.1); 0.319
	Ca(mmol/L)		-0.8(-6, 4); 0.744	-0.8(-2, 0.5); 0.237	0.1(-0.1, 0.3); 0.245	-0.05(-0.2, 0.1); 0.451	-1(-2.3, 0.33); 0.142	-0.1(-0.4, 0.23); 0.680
	Phos (mmol/L)		4.1(-1.1, 9);0.121	-0.2(-1.5, 1.2); 0.816	0.2(0.01, 0.4); **0.035**	-0.1(-0.2, 0.1);0.327	-0.4(-1.7, 1.1); 0.610	0.05 (-0.3, 0.4); 0.743

Data was analyzed using multi linear regression and presented as Beta coefficient (95% Confidence Interval); statistically significant results ($p<0.05$) are highlighted in bold. 25(OH) D: 25-Hydroxyvitamin D; TT3: Total Triiodothyronine; FT3: Free Triiodothyronine; TT4: Total Thyroxine; FT4: Free Thyroxine; TSH: Thyroid-Stimulating Hormone.

6. Discussion

This study aimed to compare the serum vitamin D and thyroid function test levels between newly diagnosed BC patients with apparently healthy controls at TASH in Addis Ababa, Ethiopia. In the control group, 10.1% had thyroid-related pathology (four with subclinical hypothyroidism and three with subclinical hyperthyroidism). Among the BC cases, 15.9% had thyroid dysfunction (five with subclinical hyperthyroidism, five with subclinical hypothyroidism, and one with clinical hypothyroidism). No clinical hyperthyroidism was observed in either group. Only 14.5% cases and 27.5% individuals from the control group achieved sufficient 25 (OH) D concentrations. A significant mean difference in serum TT3 concentration and a significant median difference in the concentrations of serum E2, Prog, and PRL between the case and control groups were observed.

The mean TT3 concentrations were significantly lower in the BC patients (1.2 ± 0.28ng/mL) than in the control group (1.4±0.19ng/mL) ($p<0.001$). This finding aligns with previous study in 2024 (62). However, our findings differs from the a study conducted in 2014 in which they observed a significant increase in TT3 levels among BC patients compared to controls (49). The observed discrepancies may be influenced by genetic predispositions affecting thyroid hormone metabolism, differences in iodine intake, or thyroid hormone resistance mechanisms in addition to analytical methods. Thyroid hormone transport and conversion efficiency may also differ between populations due to genetic polymorphisms in deiodinases and thyroid hormone receptors, which were not assessed in this study.

In this study, a statistically significant lower FT3 levels ($P<0.001$) was observed among the case group (median=2.7pg/mL, IQR=2.3–3.1pg/ml) compared to controls (median=3.0 pg/mL, IQR =2.8–3.3pg/mL). The results of the current study was similar to Takatani O *et al.* findings who observed mean level of serum FT3 significantly lower in BC patients compared to the control group regardless of menstrual status (63). However, our study was different from a study conducted in 2017 and in 2018 that findings showed no discernible change between the control and BC in levels of FT3 (38,43). Moreover, the current finding is also different from Saraiva PP *et al.* study which demonstrated a statistically significant higher concentration of FT3 level in BC patients than in the control groups (26). The observed disparities could be caused by differences in menopausal status, differences in precision and sensitivity of laboratory

procedures, different sample sizes, or different study designs. Interestingly, all of the studies that were compared had smaller sample sizes than ours. For example, Jarari AM *et al.* used enzyme immunoassays for their analytical processes and a prospective observational study design, which may have contributed to the observed disparity in results.

The mean TT4 and FT4 values in this study in the case and control groups did not differ significantly ($p>0.05$). The current study also shows no significant difference in median TSH levels between BC patients and healthy controls ($P=0.416$). This result is consistent with the findings of 2024 study (62), showing that no significant associations of TT4, FT4,and TSH levels between BC patients and control groups. However, the current result differs from (41) findings, which reported a statistically significant increase in TT4 concentrations among BC patients as compared to control groups .

Moreover, the current study also differs from (51) which evidenced lower serum FT4 and higher TSH in the case than in the control groups. The disparity may be explained by methodological variations, However another study in 2020 reported BC patients had significantly lower TSH and higher FT4 concentrations than the control groups (61). Another finding in 2017 (46) showed higher FT4 levels in the BC patients compared to the control groups. While methodological factors could contribute to some discrepancies, variations in genetic susceptibility, iodine status, and the presence of thyroid autoantibodies could also play a role. Some studies suggest BC patients may have underlying thyroid autoimmunity, which can alter thyroid hormone levels independently of cancer progression.

In the current study, the case group had significantly higher levels of serum reproductive tests (E2, Prog, and PRL) compared to healthy controls. The median E2 level in the case (11.7pg/mL, IQR 5.0–40.7) was notably higher than in the control group (5.0pg/mL, IQR 5.0–5.0, $p<0.001$). Similarly, in this study, elevated serum Prog and PRL levels were observed in BC patients, with significant median differences compared to controls ($p<0.001$ and $P=0.024$, respectively). These results are supported by the study findings of Trehan AS *et al* (64) who found that serum PRL, and E2 levels were considerably higher in patients than in controls. Furthermore, Trabert B. *et al.* revealed in 2020 that elevated circulating Prog levels were risk factors for BC (65). Hussein DA *et al.* showed that BC patients had lower levels of Prog and E2 than the controls, which is in contradiction to the findings of the current study (66). The discrepancies in PRL and Prog levels with studies such as Hussein DA et al. may be linked to unaccounted variations in menstrual

cycle phase, menopausal status, or genetic polymorphisms in estrogen and progesterone receptors.

Furthermore, this outcome differed from that of Tadesse E *et al.* (67) and Saraiva PP *et al.* (25) who found no significant difference in E2 levels between BC patients and controls. The possible cause fir the difference may due to they obtained fasting blood samples, but our analysis employed no fasting samples, even though our study had a higher sample size. Hormon al fluctuations in premenopausal versus postmenopausal women could also account for these discrepancies. There is a lower mean serum Prog levels in BC patients compared to controls a study done by Tadesse E *et al* (67) The probable cause might be due to a larger sample size in this study. Additionally, factors like BMI and lifestyle may influence hormone levels, explaining the variation in findings.

In the current study, the difference in median 25(OH) D concentration between BC patients and the control group was statistically significant (*p=0.043*); patients with BC showed slightly lower median levels of 25(OH) D compared to the control group. This result is in line with a research conducted in 2018 (68) and in 2022 (69) in which they reported serum 25-OH vitamin D levels of cases were significantly lower compared to the control group. In contrast to the current study finding, the previous studies such as Kim JA *et al,*2020 and Eliassen AH *et al,* 2016 findings (62 ,63) reported mean serum 25(OH)D level was not statistically significant among BC patients and control groups. The difference may be influenced by the LC-MS/MS method (Kim et al.), along with lifestyle, location, genetics, and melanin variation.

In this study, vitamin D status was assessed using different cutoff values as less than 10ng/mL severe vitamin D deficiency (SVDD), 10-20 ng/mL moderate vitamin D deficiency (VDD), and >20 ng/mL as normal (sufficient). The results showed that both groups had a significant prevalence of deficiency: 85.5% of the BC patients and 72.4% of the controls. Interestingly, patients had a higher prevalence of SVDD (26.1%) than controls (11.6%). The current finding is comparable with the results reported in 2020 reported a high prevalence of SVDD (27.9%) in the case group and lower (9.3%) in the control group (70). However, the proportion of SVDD (26.1%) was less in the present study as compared to Ahmed JH *et al* 2019, finding in which the proportion of SVDD was 41.1% newly diagnosed BC women in Ethiopia (39). The observed disparity may be caused by variations in study design which is case control for Ahmed JH *et al.* and comparative cross-section in this study. Furthermore, Ahmed used more sample than this

study. On the other hand, the current study finding had a higher SVDD than the previous study findings reported from Australia (only in 10.3% of BC patients) (73). The observed disparities may be caused by sample collection season, BC types and stages, variations in geographic location (and thus exposure to sunshine), cultural variables, or dressing styles that restrict exposure to sunlight.

Our multiple linear regression analysis of factors influencing 25(OH) D and thyroid function test levels (TT3, FT3, TT4, FT4, and TSH) revealed several significant associations.

Stage II breast cancer showed decreased FT3 and FT4 levels, while Stage III had a significant positive association with TT3 and FT4, consistent with (74)finding. In Stage IV, FT4 and FT3 levels were 4% and 40% lower than controls, respectively. Invasive ductal carcinoma cases had lower TT3, FT3, and FT4 levels, while invasive lobular carcinoma cases showed reductions in all thyroid function tests except TT4. These findings suggest that advanced breast cancer may disrupt endocrine function, aligning with previous research by (75) and (76), which reported a strong negative correlation between thyroid function and aggressive breast cancer. A study conducted in 2021 also reported lower FT3 in patients with late-stage cancer, linking it to no thyroidal illness syndrome (NTIS), a condition where peripheral conversion of T4 to T3 is impaired (77). However, this differs from the findings of Study (78), which found no significant association between thyroid function tests and invasive ductal carcinoma or invasive lobular carcinoma types. These variations may be due to differences in study populations, sample sizes, methodologies, or underlying patient characteristics, such as genetic predisposition and hormonal influences.

Similarly, 25(OH) D levels were lower in Stage IV breast cancer ($\beta = -1.9$, $p = 0.002$) and IDC cases (0.3 units lower, $\beta = -0.3$, $p = 0.003$) compared to the control group, consistent with previous studies (79,80), which suggest that vitamin D deficiency is associated with advanced breast cancer, higher tumor grades, and larger tumor size. However, this contrasts with the findings of Imtaiz *et al*, who reported no significant association between serum vitamin D levels and tumor characteristics, including histology, grade, and stages of BC (55). Variations in these findings may result from unmeasured confounders, including diet, physical activity, and genetic factors, which can influence vitamin D metabolism and its role in cancer progression.

7. Strength and Limitation of the study

7.1. Strength of the Study

This study provides a comprehensive analysis of thyroid function and vitamin D status in breast cancer patients, offering a holistic view of endocrine disruptions. The inclusion of a healthy control group enhances the validity of observed differences, while stage-wise stratification allows for a deeper understanding of hormonal variations. A robust statistical approach, including multiple regression analysis, strengthens the reliability of associations. Additionally, standardized laboratory testing and data collection minimize information bias and period effects, ensuring the study's credibility.

7.2 Limitation of the Study

This study has limitations. The BC status of control participants was not confirmed via mammography, potentially introducing selection bias and misclassification. Vitamin D levels were measured using immunoassay instead of the gold-standard LC/MS/MS, which may lead to underestimation. The cross-sectional design limits causal inferences on thyroid dysfunction and vitamin D deficiency in breast cancer progression. While the sample size is larger than in some prior studies, it still affects generalizability. Additionally, a lack of relevant literature in our setting poses a challenge for comparison.

8. Conclusion and Recommendation

TT3, FT3, and 25(OH) D levels were significantly lower in BC patients than in healthy controls, highlighting their potential as disease markers. The high prevalence of vitamin D deficiency in both groups emphasizes the need for preventive interventions. Additionally, reproductive hormone levels (E2, PRL and Prog) were significantly higher in BC patients. Multiple regression analysis revealed significant variations across cancer stages, with advanced stages, particularly Stage IV, showing lower FT3, FT4, and 25(OH) D levels, indicating disrupted endocrine regulation. Routine screening of thyroid function and vitamin D levels in breast cancer patients may help in early detection and management of endocrine imbalances. Targeted vitamin D supplementation is recommended, especially for patients with severe deficiency in advanced stages. Future longitudinal studies are needed to establish causal links between thyroid dysfunction, vitamin D deficiency, and BC progression.

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Annexes

Annex I: Information sheet (English)

CODE _____

This informed consent form is for newly diagnosed breast cancer patients admitted to TASH.

PI's full name: Mehari Meles Lema

• Cell phone address: +251 924862990

• Email Address: meharimeles89@gmail.com

Introduction: Hello! I'm …… from Addis Ababa University and I'm here with other colleagues to research Iron profiles in breast cancer patients. I'm going to inform you and ask you to participate in this research project. You can decide whether or not to take part in the study.

Aim of the study: To assess serum Vitamin D Concentration and Thyroid Function Profiles among female breast cancer patients at TASH.

Type of Research Intervention: This research will involve your participation in anthropometry measurements, and giving blood specimens (for this research). We will make 5 minutes interview with you to collect little clinical information.

Voluntary Participation: This study just asks for your voluntary involvement. Whether or not you participate is entirely up to you. Nothing will change in your daily life if you decide not to take part in this study. Any medical care you receive is unaffected by your choice not to volunteer.

Risks and discomforts: When collecting blood, there may occasionally be minor bleeding and minor discomforts or pains, which can be managed by trained health professionals collecting the data.

Benefits: Although there will be no direct benefit to you, your involvement will help us to assess serum Vitamin D Concentration and Thyroid Function Profiles among breast cancer patients.

Incentive: There won't be any additional compensation for your participation in this study.

Confidentiality: The data acquired about your health and the lab results will be kept completely confidential. Individual information will remain private and findings will only be released in aggregate form.

Right to Refuse or Withdraw: If you want to do so, you are always free to revoke your consent to the study for any reason.

Annex II: Consent Form (English Version)

Code of study subject _____

I have been informed about a study plan that is entitled "Assessment of Serum Thyroid Function Profiles and Vitamin D among Females with Breast Cancer in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia" and for this purpose some information and blood samples will be taken from me. I was informed about the objectives of this study. The sample would be collected using the standard method for laboratory investigation; however, drawing blood can be painful.

I've also been told that the questionnaire's data will be kept private. In addition, I am fully aware of my rights to refuse to participate in the study, hold onto information, and withhold my consent. I have been told that my laboratory results will be shared with me as soon as they are available, and if they contain any abnormal indicators, I will be sent to the proper facility for additional evaluation and care.

I freely gave the researcher my informed agreement to utilize the sample I provided for the investigation since I was fully aware of the circumstances. I've had the chance to inquire about it as well, and I've gotten answers that satisfy me.

Participant's signature: _____ Investigator's signature: _____

Annex III: Questionnaire (English Version)

Questionnaire No ☐ date……

Part I: Socio-Demographic Characteristics

1. Age:
2. Marital status: (a) Single (b) Married (c) Divorced (d) separated
3. Educational Status: (a) no formal education (c) Secondary or less (d) Diploma and above
4. Occupation: (a) Farmer (b) Government employee (c) Private Sector employee (d) Self-employment (e) Unemployed
5. Parity number _____
6. BMI: _____
7. Menopausal status: yes ☐ no ☐
8. Do you Drink alcohol yes ☐ no ☐
9. Have you smoke cigarettes in your life at least 100 cigarettes? Yes ☐ no ☐
10. Chewing chat yes☐ no ☐
11. Do you use contraceptives, in your life? yes ☐ no ☐
12. How much time you are exposed to sunlight (in minute) ?

A. Less than 30minute/day B. Greater than 30minutes/ day

13. Do you use sunscreen? A. Yes B. No

Part II: Clinical characteristics

1. Stage of breast cancer (a) Stage I (b) Stage III (c) Stage II (d) Stage IV
2. Types of breast cancer (a) Invasive ductal (b) Invasive stromal sarcoma (c) Noninvasive ductal
3 Family history with breast cancer yes ☐ no ☐

Annex IV - Information sheet to participants (Amharic version)

የዋና መርማሪ ሙሉ ስም፡ መሓሪ መለስ ለማ

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አባሪ 1፣ የተሳታፊዎች መረጃ ቅጽ

ሰላም ጤና ይስጥልኝ……...እባላለሁ_____የስራ ባልደረቦቼ ደግሞ_____ እና_____ይባላሉ። በአዲስ አበባ ዩኒቨርሲቲ ጤና ሳይንስ ኮሌጅ በክልካል ኬሚስትር ትምህርት ክፍል የሁለተኛ ዲግር ተመራቂ ተማሪ ስንሆን የዚህ ጥናት ራዕስ “Assessment of Serum Vitamin D and Thyroid Function Tests in female breast cancer patients attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia” የሚል ነዉ። እባክዎ በዚህ ጥናት ዉስጥ ለመሳተፍ ከመስማማትዎ በፍት ቀጥለዉ የምገኝዉን ሀሳብ በጥሞና ያንብቡና ግልፅ ያልሆነ ካለ ይጠይቁ። የእርስዎ በዚህ ጥናት ላይ የሚኖርዎት ተሳትፎ ሙሉ በሙሉ በግል ፈቃደኝነት ላይ የተመሰረተ ሲሆን ለመሳተፍ አልያም ላለመሳተፍ ቢወስኑ እንኳ በዚህ ሆስፒታል ዉስጥ የሚያገኙት ማንኛዉም አገልግሎት አይቋረጥም። የጥናቱ ተሳታፊ ከሆኑ የሚጠበቅበዎት በዚህ ጥናት ለመሳተፍ ፈቃዶዎ ከሆኔ አንዳንድ ቃለ መጠይቆችን ለመመለስና በስተመጨረሻ ሁለት የሻይ ማንክያ ያክል የደም ናሙና ለመስጠት መስማማት ይጠበቅቦታል። በዚህ ጥናት መሳተፍ የሚያስገኝዉ ጥቅም ይህ ጥናት የሁለተኛ ዲግር መመረቂያ ፅሁፍ እንደመሆኑ መጠን የምከፈልዎት ገንዘብ ባይኖርም በዉጤቱ ተጠቃም ነዎት። ምክንያቱም የእርስዎ ተሳትፎ በእርስዎና በወገነዎ ደም ዉስጥ የሚገኝ የአይረን መጠን ከጡት ካንሰር ጋር ያለዉን ተያያዥነት እና ስጋት ለጤና ባለሙያዎች ስለሚያሳይ እንደ እርሶ ያሉ ታካሚዎችን በአማራጭ መንገድ ይበልጥ እንዲረዱት ያግዛል። በዚህ ጥናት መሳተፍ የሚያስክትለዉ ጉዳት መጠነኛ የደም ናሙና ከእጆዎ በምወሰድበት ጊዜ አነስተኛ የህመም ስሜት ሊኖር ይችላል። ነገር ግን ናሙናዉ የሚወሰደዉ በከፍተኛ ጥንቃቄ ልምድ ባለዉ ባለሙያ ስለሆኔ ህመሙ በጥቂት ደቂቃዎች ዉስጥ ይተዋል።

አባሪ 2: የተሳታፍዎችን ምስጥር ስለመጠበቅ

ይህ የሚሰጡን ግለሰባዊ መረጃ ምስጢራዊነቱ የተጠበቀ ነው። ይህም መረጃ በኮምፒዩተር በምስጢር ከተመዘገበ በኋላ ስምዎት አይጠቀስም። እናም በምንም ዓይነት መንገድ ሊታወቅ አይችልም።በኮምፒዩተር ዉስጥ ያለዉ መረጃም በምስጢር ኮድ ታስሮ ይቀመጣል። ወረቀቶቹም በምስጢር ይቀመጣሉ። ይህን መረጃ የሚሰጡት ያለምንም ግዴታ በሙሉ ፈቃደኝነትዎ ነዉ። ጥናቱ ከተጀመረ በኋላ በማንኛውም ጊዜ ተሳትፎዎን የማቋረጥ ሙሉ መብት ይኖርዎታል። በጥናቱ ላይ መሳተፍ በሆስፒታሉ ካለዎት ግንኙነት ጋር ተፅእኖ የለዉም:: በጥናቱ ላይ ለመሳተፍ የሚፈልጉ ከሆነ ወይ የተያያዘውን መረጃ ስምምነት መፈረም ወይም ደግሞ መስማማትዎን ጥናቱን ለሚሰበስበዉ ሠዉ ይንገሩ እነሱም በርስዎ ስም ይፈርማሉ።

ከምስጋና ጋር

የጥናቱ ተሳታፊ መለያ ቁጥር _____ፊርማ_____

የመረጃ ሰብሳቢው ሙሉ ስም_____ፊርማ_____

አባሪ 3: ፈቃደኝነትን የሚያረጋግጥ ቅጽ

የጥናቱ ተሳታፊ መለያ ቁጥር _____

"ስለ አይረን መጠን ከጡት ካንሰር ጋር ያለዉን ተያያዥነት, በኢትዮጵያ" የአይረን ሁኔታ በጡት ካንሰር ህመምተኞች ላይ ለመለየት ስለሚረዳው የማጥኛ ዕቅድ ተነግሮኛል:: ለዚህም ጥቂት መረጃ እና የደም ናሙና ይወሰዳል:: የዚህ ጥናት ዓላማ ለኔ ተብራርቷል:: የናሙና ማሰባሰቡ የተለመደውን የምርመራ ሂደት ይከተላል። ነገር ግን ከደም መሰብሰብ ጋር የተያያዘ ህመም ሊኖር ይችላል::

በመጠይቁ ውስጥ የተካተቱ ሁሉም መረጃዎች በምሥጢር እንደሚጠበቁ ተብራርቶልኛል። ከዚህም በላይ የመረጃ መብቴን የመጠበቅ, የመተባበር መብቴን እና ከምርመራው ራሴን የማውጣት መብቴ እንደሚጠበቅ ተነግሮኛል።ውጤቱ ዝግጁ በሆነ ግዜ የላቦራቶሪ ውጤቶች እንደሚገለጹልኝ ተነግሮኛል:: በምርመራውም የህመም ምልክት ልሆን የሚችል ነገር ከተገኘብኝ ለተጨማሪ ምርመራ እና ሕክምና እንዳገኝ ወደ ተገቢ ቦታ እንደሚገናኝ ተነግሮኛል::ከዚህም በላይ ስለ ጉዳዩ ጥያቄዎች የመጠየቅ እድል አግኝቻለሁ እና በተሰጡኝ መልስ ረክቻለሁ።

Annex V: Questionnaire (Amharic Version)

አባሪ 4፣ የመጠይቅ ቅጽ ክፍል 1፣ ግለሰባዊ መረጃዎ

1. ዕድሜ---------

2. የጋብቻ ሁኔታ ሀ. ያላገባ (ች) ለ. ያገባ (ች) ሐ. በሞት የተለያየች መ. በፍች የተለያየች

3. የትምህርት ሁኔታ ሀ. አልተማርኩም ለ. ሁለተኛ ደረጃ ሐ. ድኘሎምና ከዛ በላይ

4. የሥራ ሁኔታ ሀ. ገበረ ለ. የመንግስት ሰራተኛ ሐ.የግል ድርጅት መ. የቤት እመቤት ሠ. ስራ አጥ

5. የእርግዝና ቁጥር _____

6. BMI (Kg/m2 _____

7. እርጣት ደረሰዋል? ሀ. አዎ ለ. አይደለም

8. ከዚህ በፍት ከቤተሰብ/ከቤተ ዘመድ መካከል ካንሰር የታመመ አለ? ሀ. አዎ ለ. አይ

9. አልኮል ጠጥተው ያውቃሉ? ሀ. አዎን ለ. አልጠጣም ሐ. አቁሚያለሁ

10. ሲጋራ አጭሰው ያውቃሉ? ሀ. አዎን ለ. አላጤስኩም ሐ. አቁሚያለሁ

11. ጫት ይቅማሉ? ሀ. አዎን ለ. አልቅምም ሐ. አቁሚያለሁ

12. የቤተሰብ እቅድ ይጠቀማሉ ሀ. አዎን ለ. አይ

13. በወር ስንተ ዓሳ ይመገባሉ? ሀ. ከ 30 ደቂቃ በታች ለ. ከ 30 ደቂቃ በላይ

14. በቀን ምን ያክል ደቂቃ ጸሀይ ያገኛሉ? _____

15. ጸሀይ ለመከላከል የሚቀባ ቅባት ይጠቀማሉ? ሀ. አዎ ለ.አይደለም

Annex-VI: procedures (SOP) for Serum Vitamin D and thyroid function tests

Blood Sample Collection

Blood samples were collected from the anti-cubital vein of the arm by using syringes after proper antisepsis with alcohol and sterile cotton swabs. Then the blood from each participant was transferred to a serum separator tube and allowed to stand for 30 minutes. The serum was separated by centrifugation at 4000rpm for 5 minutes. All the medical equipment used for blood collection were safe and sterile.

Procedure for serum separation

1. 5 ml whole blood was drawn into a serum separator tube containing no anticoagulant.

2. It was kept in the upright position at room temperature for 30-45 min to allow clotting.

3. It was centrifuged for 5 min at the manufacturer's recommended speed of 4000 rpm.

4. The serum was carefully aspirated at room temperature and pooled into a centrifuge tube, taking care not to disturb the cell layer or transfer any cells. A clean pipette for each tube was used.

5. Serum was inspected for turbidity and hemolysis.

6. Aliquot into cryo-vials and store at –80°C. The cryo-vials were labeled with the participant's identification number.

Procedure for performing Thyroid-stimulating hormone (TSH, thyrotropin)

Clinical Utility: Thyroid-stimulating hormone (TSH, thyrotropin) is a glycoprotein with a molecular weight of 30000daltons and two subunits. TSH-specific immunological and biological information is carried by the subunit, whereas species-specific information is carried by the chain, which has an identical amino acid sequence to the chains of LH, FSH, and hCG. TSH is produced by particular basophil cells in the anterior pituitary gland and has a circadian secretion pattern. The fundamental regulatory mechanism for thyroid hormone biological action is the hypophyseal production of TSH (thyrotropic hormone).TSH stimulates all phases of thyroid hormone production and secretion; it also

has a proliferative effect. TSH determination is the first step in thyroid diagnosis. Even minor changes in the quantities of free thyroid hormones cause significantly larger opposing changes in TSH levels.

As a result, TSH is a very sensitive and specific metric for assessing thyroid function, and it is especially useful for the early detection or exclusion of abnormalities in the central regulatory circuit involving the hypothalamus, pituitary, and thyroid.

Test principle: Sandwich principle. The total duration of the assay: 18 minutes.

▪ 1st incubation: 50µL of the sample, a biotinylated monoclonal TSH-specific antibody, and a monoclonal TSH-specific antibody labeled with a ruthenium complex react to form a sandwich complex.

▪ 2nd incubation: After the addition of streptavidin-coated micro-particles, the complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

▪ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with Pro-Cell/Pro Cell-M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is an instrumentspecifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

Procedure for Performing Triiodothyronine(T3)

Clinical Utility: Triiodothyronine (T3) is the hormone principally responsible for the development of the effects of the thyroid hormones on the various target organs. T3 (3, 5, 3'Triiodothyronine) is mainly formed extrathyroidal, particularly in the liver, by enzymatic 5' deiodination of T4. Accordingly, the T3 concentration in serum is more a reflection of the functional state of the peripheral tissue than the secretory performance of the thyroid gland. A reduction in the conversion of T4 to T3 results in a decrease in the T3 concentration. It occurs under the influence of medicaments such as propranolol,

glucocorticoids, or amiodarone and in severe non-thyroidal illness (NTI), and is referred to as “low T3 syndrome”. As with T4, over 99 % of T3 is bound to transport proteins. However, the affinity of T3 to them is around 10-fold lower. The determination of T3 is utilized in the diagnosis of T3 hyperthyroidism, the detection of early stages of hyperthyroidism, and for indicating a diagnosis of thyrotoxicosis factitia.

Principle: Competition principle. The total duration of the assay: 18 minutes.

▪ 1st incubation: 30µL of sample and a T3-specific antibody labeled with a ruthenium complex; bound T3 is released from the binding proteins in the sample by ANS.

▪ 2nd incubation: After the addition of streptavidin-coated microparticles and biotinylated T3, the still-free binding sites of the labeled antibody become occupied, with the formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

▪ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is an instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Procedure for performing thyroxine (T4)

Clinical Utility: The hormone thyroxine (T4) is the main product secreted by the thyroid gland and is an integral component of the hypothalamus-anterior pituitary thyroid regulating system. It has the function of metabolically influencing metabolism. Thyroxine is formed in a coupling reaction from two DIT molecules (3,5-diiodotyrosine) in the thyroid gland. It is stored bound to thyroglobulin in the lumina of the thyroid follicles and is secreted as required under the influence of TSH. The major part (> 99 %) of total thyroxine (T4) in serum is present in the protein-bound form. As the concentrations of the transport proteins in serum are subject to exogenous and endogenous effects, the status of the binding proteins must also be taken into account in the assessment of the thyroid hormone concentration in serum. If this is ignored, changes in the binding proteins

(e.g. due to estrogen-containing preparations, during pregnancy, or in the presence of a nephrotic syndrome, etc.) can lead to erroneous assessments of the thyroid metabolic state. The determination of T4 can be utilized for the following indications: the detection of hyperthyroidism, the detection of primary and secondary hypothyroidism, and the monitoring of TSH-suppression therapy.

Principle: Principle Competition principle. The total duration of the assay: 18 minutes.

▪ 1st incubation: 15µL of sample and a T4-specific antibody labeled with a ruthenium complex; bound T4 is released from binding proteins in the sample by ANS.

▪ 2nd incubation: After the addition of streptavidin-coated microparticles and biotinylated T4, the still-free binding sites of the labeled antibody become occupied, with the formation of an antibody-hapten complex. The entire complex becomes bound to the solid phase via the interaction of biotin and streptavidin.

▪ The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is an instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Procedure for performing Vitamin D total

Clinical Utility: Vitamin D is a fat-soluble steroid hormone precursor that is mostly synthesized in the skin by sunshine exposure. Vitamin D is physiologically inactive and must be hydroxylated twice in the liver and kidney to generate the biologically active 1,25dihydroxyvitamin D. Vitamin D is necessary for bone health. In youngsters, severe deficiency causes rickets, a bone deformity. Milder degrees of insufficiency are thought to result in less efficient calcium utilization. Muscle weakness is caused by vitamin D insufficiency; among the elderly, the influence of vitamin D on muscle function has been linked to an increased risk of falling.Secondary hyperparathyroidism is frequently caused by a vitamin D shortage. Elevated parathyroid hormone levels, particularly in senior vitamin D-deficient persons, can cause osteomalacia, accelerated bone turnover,

decreased bone mass, and an increased risk of bone fractures. Low 25hydroxyvitamin D levels are also linked to poorer bone mineral density. The findings, when combined with additional clinical data, could aid in the assessment of bone metabolism. Vitamin D has been demonstrated to influence the expression of over 200 different genes thus far. Diabetes, various types of cancer, cardiovascular disease, autoimmune illnesses, respiratory diseases, and innate immunity have all been associated with insufficiency.

Principle: Competition principle. The total duration of the assay: 27 minutes.

▪ 1st incubation: By incubating the sample (15 µL) with pretreatment reagents 1 and 2, bound 25-hydroxyvitamin D is released from the VDBP.

▪ 2nd incubation: By incubating the pretreated sample with the ruthenium-labeled vitamin D binding protein, a complex between the25-hydroxyvitamin D and the ruthenate VDBP is formed. A specific unlabeled antibody binds to 24,25-dihydroxyvitamin D present in the sample and inhibits cross-reactivity to this vitamin D metabolite.

▪ 3rd incubation: After the addition of streptavidin-coated micro-particles and 25-hydroxyvitamin D labeled with biotin, unbound ruthenate-labeled vitamin D binding proteins become occupied. A complex consisting of the ruthenate vitamin D binding protein and the biotinylated25-hydroxyvitamin D is formed and becomes bound to the solid phase via the interaction of biotin and streptavidin.

▪ The reaction mixture is aspirated into the measuring cell where the micro-particles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

▪ Results are determined via a calibration curve which is an instrument specifically generated by 2-point calibration and a master curve provided via the reagent barcode or e-barcode.

Procedure for performing calcium total

Test principle

The method was based on calcium-NM-BAPTA complex formation through the reaction of Calcium ions with 5-nitro-5-methyl-BAPTA (NM-BAPTA) under alkaline pH (around pH of 10) then the complex reacts with EDTA (Roche Diagnostics Corporation., 2014).

Alkaline pH

Ca^{2+} + NM-BAPTA ⟶ calcium-NM-BAPTA complex

Calcium-NM-BAPTA complex + EDTA ⟶ NM-BAPTA + calcium EDTA complex

The change in absorbance, which is directly proportional to the calcium concentration, is measured photometrically at 340 nm (Bourguignon *et al.*, 2014). COBAS 6000 analyzers automatically calculate the analyte concentration of each sample.

Procedure for performing Serum phosphate

Test principle

Endpoint method with sample blanking Inorganic phosphate reacts with ammonium molybdate in the presence of sulfuric acid to form ammonium phosphomolybdate complex having the formula $(NH_4)_3[PO_4(MoO_3)_{12}]$. The complex was determined photometrically in the ultraviolet region at 340 nm wavelength. Accelerator can be added to get rapid reaction rate and more precise result will be gotten by application of sample blanking. COBAS 6000 analyzers automatically calculate the analyte concentration of each sample (Roche Diagnostics Corporation., 2016).

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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Date of submission: _____

This thesis has been submitted with our approval as advisors.

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Advisor: Mekdes Alem (MSc)

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Date: _____

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Co-advisor: Dr. Habteyes Hailu Tola

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