



UNIVERSITAS INDONESIA

**SERUM LEVEL OF VITAMIN D IN PATIENTS WITH
CANCER IN COMPARISON TO HEALTHY CONTROLS IN
RUMAH SAKIT CIPTO MANGUNKUSUMO, JAKARTA**

RESEARCH REPORT

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**FAKULTAS KEDOKTERAN
PROGRAM STUDI PENDIDIKAN DOKTER
JAKARTA
JUNI 2015**



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RUMAH SAKIT CIPTO MANGUNKUSUMO, JAKARTA**

RESEARCH REPORT

A final project report presented to Universitas Indonesia as a partial prerequisite
to obtain Bachelor of Medicine

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JAKARTA
JUNI 2015**

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I hereby certify that the material presented in this research project report
(undergraduate) is my original work and that all the resources, whether cited or
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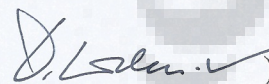
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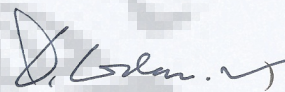
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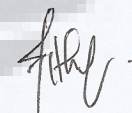
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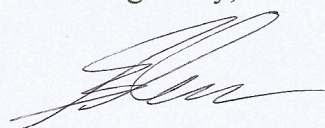
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ABSTRAK

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Program Studi : Pendidikan Dokter
Judul : Level serum vitamin D pada pasien penderita kanker dalam perbandingan dengan kontrol sehat di Rumah Sakit Cipto Mangunkusumo, Jakarta

Beberapa penelitian terbaru menunjukkan vitamin D tidak hanya berperan dalam homeostasis untuk kalsium dan fosfor, namun juga berperan dalam pengaturan sistem imun. Telah diketahui secara umum bahwa kekurangan vitamin D dapat menyebabkan penyakit rakitis. Namun ternyata penelitian-penelitian baru mengusulkan bahwa hal ini juga menjadi faktor risiko pembentukan dan perkembangan kanker. Kanker dapat dianggap sebagai masalah kesehatan publik di Indonesia dimana terlihat adanya peningkatan kecenderungan penyakit tidak menular ini pada beberapa tahun terakhir. Penelitian ini bertujuan untuk mendalami pengetahuan perihal tingkat vitamin D pada serum pasien kanker di Rumah Sakit Cipto Mangunkusumo, Jakarta dan meninjau apakah tingkat vitamin D mempengaruhi jumlah kadar limfosit mengingat kaitannya pada sistem imun. Tingkat vitamin D pada 35 pasien kanker yang belum mendapatkan pengobatan dan 39 kontrol sehat yang diukur melalui *immunoassay* dan data limfosit yang diperoleh dari rekam medis pasien. Semua data yang didapat kemudian di analisa untuk meninjau hubungan antara tingkat vitamin D dengan jumlah kadar limfosit. Perbedaan antara tingkat vitamin D pada serum pasien kanker dan kontrol sehat tidak signifikan. Selain itu, jumlah kadar limfosit juga tidak menunjukkan perbedaan signifikan terhadap kategori status vitamin D. Kedua kondisi tersebut telah dievaluasi dalam aspek statistik dan eksperimental. Tidak ada perbedaan yang signifikan dalam aspek statistik dalam rata-rata tingkat vitamin D pada serum pasien kanker dan kontrol sehat, dimana hasil pada pasien kanker lebih tinggi dari kontrol sehat (17.93 ± 10.81 ng/mL vs. 15.16 ± 8.10 ng/mL). Korelasi antara jumlah kadar limfosit dengan tingkat serum vitamin D tidak dapat dipastikan. Pasien kanker di Rumah Sakit Cipto Mangunkusumo, Jakarta pada umumnya kekurangan vitamin D.

Kata kunci: Vitamin D, Kanker, Sistem imun, Jumlah kadar limfosit, Indonesia

ABSTRACT

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Title : Serum level of vitamin D in patients with cancer
in comparison to healthy controls in Rumah Sakit
Cipto Mangunkusumo, Jakarta

Recent studies have revealed the roles of vitamin D that extends further than homeostasis of calcium and phosphorus, which is in the immune system that is known to have regulatory properties. It is also established that vitamin D deficiency leads to rickets, however new studies suggest that it may also pose a risk for cancer development and progression. Cancer is considered a major public health problem in Indonesia as an increasing trend is seen for this non-communicable disease in the recent years. This study aims to grasp more understanding in regards to the serum level of vitamin D of cancer patients in Rumah Sakit Cipto Magunkusumo, Jakarta and observe whether the level of vitamin D affects the lymphocyte count of cancer patients when taking the role of vitamin D the in the immune system into account. The serum vitamin D level of two independent means of 35 untreated cancer patients and 39 healthy controls are compared. Serum vitamin D levels are obtained through immunoassay and lymphocyte count is obtained through white blood cell differential count from the medical records. Analysis of the data collected is done to assess the relation of vitamin D levels and lymphocyte count. The difference between serum level of vitamin D of cancer patients and healthy controls is unexceptional. Moreover, the lymphocyte count does not show any significant difference towards the vitamin D status categories. Both of these conditions were evaluated through statistical and experimental aspect. There is no statistically remarkable difference of the mean serum vitamin D levels between patients with cancer and healthy controls, in which the former is actually higher than the latter (17.93 ± 10.81 ng/mL vs. 15.16 ± 8.10 ng/mL). Correlation between lymphocyte count and serum vitamin D level is indeterminate. Cancer patients in Rumah Sakit Cipto Mangunkusumo, Jakarta, are generally vitamin D deficient.

Keywords: Vitamin D, Cancer, Immune system, Lymphocyte count, Indonesia

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CHAPTER 1 INTRODUCTION

1.1 Background

Back in the 1650s, Glisson was able to thoroughly explain about Rickets, although a gap was still present regarding the cause of the disease.¹ For almost more than 260 years later, Hopkins suggested a cause for Rickets, which is also surprisingly turned out to be part of our dietary factor that is vitamin D.² About two decades later, vitamin D and even its precursor are able to be isolated.^{1,2} Since then, the role of vitamin D is explored further.

Cholecalciferol (vitamin D3) is not considered to be essential for inclusion in our dietary intake as it can be produced by the body through adequate exposure to sunlight.³ A more in-depth studies regarding vitamin D shows its role in maintaining homeostasis for calcium and phosphorus.^{3,4} In addition, more recent studies then discovered more roles that vitamin D takes on, which are included in the innate immune response and adaptive immune response such as barrier functioning and immunomodulatory properties respectively.⁵ These effects can be achieved when vitamin D is converted in our body in its most active form, also known as calcitriol.¹⁻⁷

In 1980s, studies have found the presence of receptors for calcitriol, vitamin D receptor (VDR), in various cell types. By means of these VDRs can calcitriol perform its function in different cells included in both innate and adaptive immune response as they bind to them and the vitamin D response elements (VDRE).⁵⁻⁷ Said reaction then leads vitamin D to utilize its capabilities as immunomodulator, anti-inflammatory, and antimicrobial effects.⁵⁻⁷

Cancer is a well-known non-communicable disease in the world, and currently it is a major public health problem in Indonesia.⁸ Based on a recent study regarding the cancer registration in Indonesia, cancer have become the 7th largest cause of death on a national survey back in 2007, accounting for 5.7% of all mortality.⁸ The prevalence of cancer in Indonesia is 4.3 per 1000 population.^{8,9} Recent studies indicates that vitamin D deficiency is highly

associated with the risk of cancer incidence, and that those with cancer also shows a severe deficiency of said vitamin.^{2,10}

Ultimately, this research aims to further explore the role of vitamin D in the immune system of cancer, their contribution towards incidence, and prognosis of current patients. In addition, hopefully this study may benefit in managing, treating, and even preventing cancer through the consideration of including vitamin D supplementations in Indonesia.

1.2 Problem Identification

1. Effect of serum vitamin D levels in the immune system of patients with cancer.
2. The lack of data available in regards to the serum level of vitamin D in cancer patients in the Fakultas Kedokteran Universitas Indonesia.
3. No information regarding the vitamin D profile of adult population in Jakarta, Indonesia.

1.3 Research Question

How does the level of serum vitamin D of patients with cancer and healthy controls differ?

1.4 Hypothesis

Serum levels of vitamin D in patients with cancer are lower compared to healthy controls.

1.5 Objective of the the Research

1.5.1 General Objective

To study, observe, and record the level of serum vitamin D in patients with cancer.

1.5.2 Specific Objective(s)

1. To determine the difference in the level of serum vitamin D between the patients with cancer and 39 healthy controls in Rumah Sakit Cipto Mangunkusumo, Jakarta

2. To investigate any possible relation between the level of serum vitamin D with the lymphocyte count of patients with cancer by checking the white blood cell differential count of the patient.
3. To observe and determine the general status of vitamin D in patients with cancer in Rumah Sakit Cipto Mangunkusumo, Jakarta.

1.6 Benefits of the Research

1.6.1 Benefits for The Community

1. To enhance the understanding of vitamin D and their part in the immune system.
2. To improve the efficacy of treatments by taking vitamin D to reckon with.
3. To raise awareness of the society regarding the significance of vitamin D in the immune system.

1.6.2 Benefits for The Institution

1. To advance further the knowledge regarding the potential role of vitamin D in correlation with immune system, especially in regards to cancer.
2. To help contribute to the Faculty of Medicine, Universitas Indonesia in achieving their goal towards becoming a world-class research university.
3. To aid in creating a research that can supplement the learning activities at the university.

1.6.3 Benefits for Researcher

1. To gain first-hand experience in carrying out a scientific research.
2. To hone the researcher's skills in terms of scientific research.
3. To start fulfilling the criteria of the Seven-Stars Doctor by being a researcher, with the hopes of fulfilling them all by the time for graduation.
4. To gain more understanding and knowledge regarding vitamin D and its correlation with vitamin D.

CHAPTER 2

LITERATURE REVIEW

2.1. Vitamin D

Vitamin D may be produced in the body through adequate exposure from sunlight, thus it is not essential to be supplied through dietary intake. It also works only on specific target tissues such as the small intestine to enhance their efficiency in absorbing dietary calcium and phosphorus; in addition to that, vitamin D also mobilized calcium and phosphorus stores from bone, thus functions to maintain serum calcium and phosphorus concentration in a certain range of level that is crucial for supporting cellular activities, neuromuscular function, and ossification of bone.^{3,4} From the characteristics mentioned previously, vitamin D it better meets the definition of a hormone rather than a vitamin, furthermore it has the molecular structure and actions that resembles a steroid hormone.^{3,4,10,11}

2.1.1 Vitamin D Synthesis and Affecting Factors

There are two sterols that serve as the precursors of vitamin D; one is in the lipids of animals (7-dehydrocholesterol, provitamin D₃) and one in plants (ergosterol).³ Each of them undergoes a photolytic ring opening when exposed to ultraviolet (UV) irradiation, specifically UVB (small band of radiation between 290 and 315 nm).⁴ Ring opening of 7-dehydrocholesterol produces a previtamin form of 7-dehydrocholesterol (previtamin D₃), which further produces cholecalciferol (vitamin D₃) after thermally stabilizing.^{4,11} The process of equilibration takes about 4 to 8 hours.^{4,11} Ergosterol ring opening yields ergocalciferol or vitamin D₂. Vitamins D₂ and D₃ require further metabolism to provide the metabolically active form of 1,25-dihydroxyvitamin D (calcitriol).^{3,4,11}

Approximately 60% of the cutaneous stores of provitamin D₃ are found in the epidermis of the adult skin, whereas the remaining 40% are in the dermis.¹¹ Around 80 to 90% of the previtamin D₃ that is formed in the skin occurs in the actively growing layers of the epidermis, including the stratum basale and stratum spinosum as they have the highest concentration of 7-dehydrocholesterol, and less than the remaining 20% or less occurs in the dermis.^{3,4}

The production of both vitamins D2 and D3 are affected by several factors such as the penetration of UVB photons, age, sunscreens, season, latitude, and time of day. Penetration of the UVB is connected with skin pigmentation, which correlates with the concentration of melanin. Melanin is our defense system against UV rays that works by absorbing them, UVB included, thus this causes a limitation in the amount of UVB that may be used for photolytic processes. Darker skin pigmentation indicates more melanin, thus further limitation in the utilizing the UVB is seen. Studies have supported this fact, by comparing the concentrations of vitamin D3 and 25-hydroxyvitamin D3 between white and black population shows that it is significantly lower in black population.^{3,4,11}

As mentioned above, age also plays a role in the photoproduction of previtamin D3 and this is related to the concentration of provitamin D3 in the skin.^{11,12} As person ages the concentration of provitamin D3 in the skin will decrease, thus providing less convertible source for previtamin D3. This matter was studied and it is proven that from the same amount of UV radiation to elderly subjects have a lower peak circulating concentrations of vitamin D, it was only 30% of that in young adults.^{3,4}

Application of sunscreens also provide a protection for the applied area towards UV radiation however, like the melanin, it also hinders the absorption of UVB that is essential for the synthesis of previtamin D3.^{3,11,12} Sunscreen with a sun protection factor (SPF) of only 8 can markedly reduce the cutaneous production of vitamin D3. Season, latitude, and time of day affect vitamin D production.^{3,4,11,12} During summer, the exposure to UV rays is longer, thus maximizing the potential for vitamin D production and an inverse relationship applies in winter because during that season, the ozone in the stratospheric layer absorbs more UVB photons.^{4,12} In certain time of day such as in the morning before around 10:00 and late afternoon after around 15:00 is advisable for elderly people to be exposed to the summer sunlight exposure as they are not too intense during those times but adequate to ensure an adequate vitamin D production.^{11,12}

2.1.2 Vitamin D Absorption, Transport, and Storage

Dietary vitamin D is incorporated with other lipids into micelles and absorbed together into the intestine by means of passive diffusion. Inside the absorptive cells, the vitamin is taken into chylomicrons, entering the lymphatic system, and subsequently enters the plasma, where it will then be delivered to the liver by chylomicron remnants or the specific vitamin D-binding protein (DBP).³

Vitamin D synthesized in the skin enters the capillary system and is transported by DBP. Vitamin D attached to CBP is delivered to peripheral tissues potentially to the colon, prostate, breast, lungs, and pancreas, while the others are stored in the liver.^{3,12}

2.1.3 Vitamin D Metabolism

The classical synthetic pathway involves 25- and 1- α -hydroxylation of vitamin D₂ and D₃, in the liver and kidneys respectively. The first occurs in the liver, vitamin D from dietary and skin production will be metabolized by vitamin D-25-hydroxylase (25-OHase, CYP27A1) into 25-hydroxyvitamin D₃ (25(OH)D₃) or calcidiol.^{3,4,12} This metabolite is the predominant circulating form of the vitamin as it can be metabolized in other tissues to maintain their normal cell proliferation and growth.¹² 25(OH)D₃ then gives negative feedback to 25-OHase.^{3,4,12} Contrary to their predominance in circulation, they are actually still idle. On that matter, it is required for 25(OH)D₃ to undergo another hydroxylation, which is in the kidney by 1- α -hydroxylase (1 α -OHase, CYP27B1) to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) or calcitriol, the most active form of this vitamin.^{3,4,12} Like 25-OHase, this hydroxylase system in the liver also receives a negative feedback from the product of the reaction in question. 1-OHase is also available in other locations in the body, however the function is also similar to calcidiol in the peripheries, which they will only be produced and used in that area. The activity of 1 α -OHase can be increased by parathyroid hormone (PTH) in the presence of low plasma concentrations of calcium, inducing an increase in the production of calcitriol.^{3,4,12}

Vitamin D activity is limited by catabolic processes, mainly mediated by 24-hydroxylase (24-OHase, CYP24A1), which transforms the calcitriol into

1,24,25(OH)₂D₃ that has a significantly lower affinity for vitamin D receptors (VDR); it will then be further degraded into products that will be excreted as calcitroic acid.^{3,4,12} The metabolization rate of vitamin D is closely regulated by limiting the metabolizing reactions of vitamin D compounds, which are the activity in the liver (CYP2R1 and CYP27A1) that is induced by low calcidiol levels and the activity in the kidneys (CYP24A1 and CYP27B1) that is induced by low calcidiol and calcitriol.^{3,4,12}

As mentioned previously regarding the distribution into peripheral tissues, the local production and degradation of calcitriol are considered to be a key factor in several types of human cancer.^{10,11,12}

2.1.4 Vitamin D Insufficiency and Deficiency

In order to define the vitamin D status in a person, an indicator is required and the best level that can be measured is the serum 25(OH)D level in the body as it represents overall vitamin D obtained through various sources such as sun exposure, diet, and conversion from fat stores in the liver.^{3,4,11,12}

The exact quantity to define the level of vitamin D, whether it is sufficient, insufficient, or deficient varies. However based on several studies such as done by Rosen (2011)¹³ and Sümbül et al. (2014)¹⁴, normal serum levels are considered as 35–55 ng/ml, the levels below 30 ng/ml are considered as insufficiency, the levels below 20 ng/ml are considered as deficiency, and the levels below 10 ng/ml are considered as severe deficiency. Thus, the range of vitamin D levels that is considered normal is 35–55 ng/ml, the range of vitamin D levels that is considered insufficient is 10–29, and the range of vitamin D levels that is considered deficient is less than 10 ng/ml.^{13,14}

Vitamin D deficiency is known to manifest as rickets in children and as osteomalacia in adults. Rickets is a disease involving impaired mineralization of growing bones and is a result of a concomitant deprivation of vitamin D, calcium, and phosphorus.^{3,4} The characteristic feature of rickets is an abnormal structure of the weight bearing bones (e.g. tibia, humerus, radius, and ulna) that may appear bowed due to their inability to handle ordinary stresses and may be accompanied by bone pain, and muscle weakness.^{3,4}

As for osteomalacia in adults, there will be generalized reductions in bone density and pseudofractures (especially of the femur, spine, and humerus) can be found.^{3,4} Due to this condition, when combined with muscular weakness, patients may have a greater risk of fractures. Prevention of osteomalacia is possible through adequate consumption of vitamin D, calcium, and phosphorus in the diet.^{3,4}

2.2 Vitamin D and The Immune System

Vitamin D has various roles in human body. It is vital in barrier functioning, modulation of immune system as well as in the synthesis of antimicrobial peptides.^{3,4} Vitamin D also works within the innate immune system and in epithelial cells of many organs including lung, GI tract, oral cavity, skin, eye and genito-urinary tract.⁵⁻⁷ Clinical studies have shown that vitamin D is also involved in prevention of infections, and is used as viral, bacterial and fungal infections treatment. Circulating 25(OH)D has been proven to enhance wound healing process.⁵⁻⁷

As briefly mentioned before, the role of vitamin D encompasses physical barrier towards injury or infection, which is the first line of defense in human body. In epithelial cells of the skin, respiratory, GI and urinary tract, the active form of vitamin D ($1,25(\text{OH})_2\text{D}_3$) up-regulates genes through the 1α -hydroxylase enzyme.⁵⁻⁷ Proteins are then encoded for the proper functioning of tight junction (e.g. occludin), adherens junctions (e.g. E-cadherin) and gap junction (e.g. connexin 43).⁵⁻⁷

2.2.1 Innate Immunity

Vitamin D also needed in innate immunity, in which it is a potent stimulator of antimicrobial peptides. In human, the antimicrobial peptides include, 4 human β -defensins (hBD-1-4), 6 human α -defensins (HNP1-4 and HD-5,6) and cathelicidin (LL-37). Synthesis of cathelicidin and some defensins relies heavily on adequate circulating 25(OH)D₃. The active hormone, $1,25(\text{OH})_2\text{D}_3$, elicits expression of cathelicidin in urogenital and bronchial epithelial cells, myeloid cell

derivatives as well as keratinocytes. Moreover, pathogens have pathogen associated molecular pattern (PAMPs) that causes activation of pathogen recognition receptors on the membranes of a cell.⁵⁻⁷ In mammals, these are called toll-like receptors (TLR), which can be found in dendritic cells, macrophages and epithelial cells. In human, TLR2/1 and TLR4 induces CYP27B1 (1 α -hydroxylase) enzyme, which in turn activates production of 1,25(OH)₂D₃. The 1,25(OH)₂D₃ then goes to vitamin D receptors (VDR) and retinoid X receptors that is followed with binding of these complexes vitamin D-response elements(VDRE).⁵⁻⁷ This leads to DNA unclocking and gene targeting that subsequently encodes relevant proteins. All these contribute in defending against invasion by bacteria, fungi or viruses. Another role of vitamin D in innate immune system is related to human monocytes, which are able to secrete hydrogen peroxide that results in elevated oxidative burst potential. The secretion of H₂O₂ is due to presence of 1,25(OH)₂D on monocytes.⁵⁻⁷

2.2.1 Adaptive Immunity

In adaptive immune function, vitamin D is needed to activate antigen specific T-cell division when there is failure of innate immune system to prevent infection. Vitamin D is also known to be involved in recruitment of other immune defense cells. Furthermore, vitamin D is able to prevent over-reaction in adaptive immune response in form of inflammatory response, which is crucial in avoiding further damage to cell or tissue.⁵⁻⁷ Vitamin D works here by limiting excessive production of pro inflammatory cytokines including IL-12 and TNF α .⁵⁻⁷

2.3 Cancer

2.3.1 Definition

Derived from the Greek word *neo* (new) and *plasma* (thing formed) means that neoplasm is the autonomous growth of tissues that deviates from the normal restraints on cell proliferation, whether it no longer shows the characteristic of their precursors, escape from their regulated cell survival and death, as well as impaired differentiation.¹⁵ Even though there is a cell that is newly and differently

formed, they may still remotely have structural resemblance to their precursor, and this helps in identifying the source and its potential behavior.¹⁶

In a sense of their space-occupying properties, solid neoplasms are called tumors. Tumors that remain localized are considered benign, whereas those that spread to distant sites are termed malignant, or cancer.¹⁵⁻¹⁷ Generally, neoplasms are irreversible, and most part of their growth is autonomous. There are some things that are essential to remember, such as: (1) neoplasms are derived from cells that normally maintain proliferative capacity. Thus, cells like mature neurons and cardiac myocytes do not give rise to tumors. (2) a tumor may express carrying degrees of differentiation, from the relatively mature structures that mimic normal tissues, to a collection of primitive cells that their origin cannot be determined. (3) the exact stimuli responsible for the uncontrolled proliferation may not be identifiable. (4) neoplasia arises from mutation in genes that regulate cell growth, death or DNA repair.¹⁵⁻¹⁷

Most of the time, malignant tumors have the capacity to kill, however benign ones spare the host. However, benign tumors can also be life-threatening in critical locations such as meningioma (benign intracranial tumor of the meninges) can kill due to the pressure it creates on the brain and myxoma (benign mesenchymal tumor of the left atrium) may cause a sudden death by blocking the mitral valve orifice.^{15,16} A similar contradiction also exist in malignancy, where certain types of malignant tumors are so idle that they pose no threat to life such as breast and prostate cancers.¹⁵

2.3.2 Classification

The classification of benign tumors is the basis for the names of their malignant variants. Benign tumors use the suffix “oma” that is preceded by reference to the cell or tissue of origin. For example, a benign tumor that is believed to derive from chondrocytes and show a resemblance to them is called chondroma, whereas it is called chondroblastoma if it resembles the precursor of the chondrocyte.^{15,16} Tumors of epithelial origin are given a variety of names based on their remarkable characteristics. Squamous epithelium may simply be termed as epithelioma but when they are branched and exophytic, it may be

termed as papilloma.¹⁷ Tumors arising from glandular epithelium are named adenoma, preceded by the location, such as thyroid adenoma or pancreatic islet cell adenoma. Benign tumors that arise from germ cells and contain derivatives of different germ layers are labeled teratoma.¹⁵

In general, malignant counterparts of benign tumors usually carry the same name and only differ in the suffix where “carcinoma” is applied to epithelial cancers and “sarcoma” is applied to those that are of mesenchymal origin.^{15,16} For instance, a malignant tumor of the stomach is a gastric adenocarcinoma. Squamous cell carcinoma is an invasive tumor of the skin or other organs lined by, as the name suggest, squamous epithelium. Naming by their origin may still be used in certain tumors, such as osteogenic sarcoma. Some tumors display neoplastic elements of different cell types but are not germ cell tumors. Such condition occurs in fibroadenoma of the breast, which is composed of epithelial and stromal elements that is benign; whereas adenosquamous carcinoma of the uterus or the lung is actually malignant.¹⁶ Tumors of the hematopoietic system are a special case, in which the suffix “emia” is used as they correlate to the blood. For instance, leukemia is a malignant proliferation of leukocytes.^{15,16}

Some secondary descriptors also refer to a tumor’s morphologic and functional characteristics, e.g. papillary is used to describe a frond-like structure, medullary is used for a soft, cellular tumor with minimal connective tissue stroma, scirrhous or desmoplastic signifies a dense fibrous stroma, whereas colloid carcinomas secrete many mucus, in which islands of tumor cells will float.¹⁶

2.3.3 Biology and Molecular Pathogenesis

Normal cell divisions are meticulously controlled in the rate and location of their proliferation and accumulation. Cancer arises with an accumulated DNA mutations within a single cell, thus when there is enough mutation that have occurred, the cell will be able to escape the regulation of growth, which leads to acquiring additional mutation that enables local invasion and a spread through vascular and lymphatic system may follow.¹⁵⁻¹⁷

Cancer production and progression requires multiple genetic events and genomic instability is essential to the generation of mutations. Generally there are

three mechanisms of genetic instability, and they are: chromosomal instability, microsatellite instability, abnormal DNA mismatch repair, and aberrant DNA methylation. Although it is not universal, it is believed that chromosomal instability, which then leads to variability in cellular karyotypes, is the most common cause. Aneuploidy, gene amplification, and loss of heterozygosity is the types of variation that arises.^{15,-17}

A defect in DNA repair also contributes to oncogenesis, one of the examples is a type of colon cancer, hereditary nonplopopsis colon cancer due to a mutated MLH1 or MSH2 DNA mismatch repair enzymes. Detection of double strand break (DSB) and intiation of the repair processes involves the ATM protein. Mutations in said enzyme and other enzymes involved in the repair of DSB.¹⁶

2.3.4 Oncogenes

Oncogenes are genes with a high potential to become cancerous, as they can escape the programmed cell death (apoptosis). Whereas a proto-oncogene, is a cell that hold the capability of turning into oncogenes due to the fact that they undergo several transformation to be a mutant of the normal gene. Several characteristics of a proto-oncogene are: production of an abnormal protein, overproduction of a normal gene product, and insensitivity to normal auto-inhibitory and regulatory constraints.¹⁵⁻¹⁷

There are three general mechanisms in which the proto-oncogenes can be activated by:

1. Mutation
2. Chromosomal translocation
3. Gene amplification

Mutations that are involved in converting proto-oncogenes to oncogenes may involve point mutations, chorosomal translocations, or deletions. Chromosomal translocation is the transfer of a portion of one chromosome to another, have been implicated in the pathogenesis of cancer such as leukemia and lymphoma. Chromosomal translocation will eventually lead to chromosomal alterations and ultimately causing an increased number of gene copies. For example, when there is a mutation and translocation may lead to an increase

expression for cells that produces an aberrant protein with very high tyrosine kinase activity, which generates mitogenic and antiapoptotic signals.¹⁵⁻¹⁷

Oncogenes can be classified into according to the roles of their proto-oncogenes in the biochemical pathways that regulate growth and differentiation, and they classification is as follows:

- Growth factors
- Cell surface receptors
- Intracellular signal transduction pathways
- DNA-binding nuclear proteins (transcription factors)
- Cell cycle proteins (cyclins and cyclin-dependent protein kinases)
- Inhibitors of apoptosis (bcl-2)

Generally, mutations that result in increased activity of a mutant gene are called gain-of-function mutations.^{15,16,17}

2.3.5 Oncogenes and Growth Factor

A few proto-oncogenes encode growth factors that stimulate tumor growth. Growth factors have two different functions, one is autocrine (acts on the cell that produces it) and the other is paracrine (acts on the the receptors of neighboring cells).¹⁶ For example of growth factor involvement in neoplastic transformation is the platelet-derived growth factor (PDGF), which is a potent mitogen for fibroblast, smooth muscle cells, and glial cells. Normally PDGF is not produced. All in all, in terms of growth factors, whether it is caused by genetic or epigenetic mechanisms, cancer cells generally produce a mixture of growth factors with autocrine or paracrine activity, thus progressing oncogenesis.¹⁷

2.3.6 Oncogenes and Growth Factor Receptors

Growth factor requires an interaction with cell surface receptors that are integral membrane proteins with tyrosine kinase activity, which interaction will then stimulate cellular proliferation. Regulation of functional responses to growth factors such as cell proliferation, differentiation, and survival depends on the expression of various growth factor receptors within a relative balance.¹⁵⁻¹⁷ When

a ligand binds to the receptors, an intrinsic kinase activity will be initiated, leading to phosphorylation of tyrosine residues on intracellular signaling molecules. Thus, due of the growth factor receptors' ability to generate potent mitogenic signals, they yield a latent oncogenic potential, which when activated, can override the normal controls of signaling pathways.^{15,17}

The most common mechanism in which they participate in oncogenesis is by overexpression of a normal receptor through enhanced activation of promoters or gene amplification. Normally receptors will turn to their resting state after binding to growth factors or other ligands, however certain mutations on the receptors can result in unrestrained activation of the receptor, independent of ligand binding.^{16,17}

2.3.7 Oncogenes and Nonreceptor Protein Kinases

Several amount of proteins with tyrosine kinase activity are loosely associated with the inner aspect of plasma membrane. They may possess tyrosine kinase activity, but they are not a part of membrane protein nor growth factor receptors.¹⁵⁻¹⁷ An example of the viral oncogene that codes for mutant forms of these protein kinases is *v-src*. The *src* enzymes are activated by most receptor tyrosine kinases and influence cell proliferation, survival, and invasiveness.¹⁵

Overall, receptor and nonreceptor tyrosine kinases are dephosphorylated as a result of inactivation by a variety of phosphatases. Mutations in the phosphatase and tensin homolog (PTEN), the product of tumor supresor gene, are linked to a variety of human malignancies.¹⁷

An example for this non-receptor protein kinsase activity is ras oncogene. Ras is an effector molecule that belongs in the signal transduction cascade that couples the activation of growth factor receptors. *Ras* proto-oncogene codes for p21, a protein that binds guanosine triphosphate (GTP) and guanosine diphosphate (GDP).¹⁶ Protein p21 is active when it binds to GTP and is inactivated when it binds to GDP. The bounded GTP is converted to GDP by intrinsic GTPase activity of p21. However, the activity of this protein is normally low, but stimulated exponentially (approximately 100-fold) by GTP-ase activating protein (GAP).^{15,16} The mutant forms of p21 are characterized by the persistence

in GTP binding, maintaining the protein to stay in its active form. This is due to a point mutation in the *ras* proto-oncogene that makes the p21 resistant to the action of GAP.

2.3.8 Oncogenes and Nuclear Regulatory Proteins

A few nuclear proteins that are encoded by proto-oncogenes are intimately involved in the sequential expression of genes that regulate cellular proliferation and differentiation.^{16,17} Numerous amounts of these proteins can bind to DNA, where they also have regulatory function towards the expression of other genes. The short-lived expression of several proto-oncogenes is necessary for the cells to pass through specific “checkpoints” in the cell cycle.¹⁵⁻¹⁷ For example, in order to leave G₀ and proceed to G₁ phase of the cell cycle, binding of PDGF to cultured fibroblast is required. Several expression of the proto-oncogenes in the early cell cycle makes the cells competent to receive the final signals for mitosis, thus they are termed competence genes.¹⁵

Competence genes, in general, plays a role in progression of the cell cycle from G₁ to S phase, genomic stability, apoptosis, and effects on cellular maturation may it be positive or negative.¹⁵⁻¹⁷ Mutations in these genes may lead to altered effects, for example elimination of a negative regulatory domain, thus prolonging its half-life and stimulating progression through G₁. Although nuclear proteins encoded by proto-oncogenes can promote cellular proliferation, in some circumstances they can also stimulate cell differentiation.^{16,17}

2.3.9 Tumor Suppressor Genes in Carcinogenesis

Accelerated cell proliferation by oncogenes is not the only factor contributing to carcinogenesis, a deficiency in normal gene product where in this case, tumor suppressor, will also play a role in cancer development because their role as negative regulatory control of cell growth is incapacitated. As opposed to gain-of-function mutations, this is called the loss-of-function mutations.¹⁵ The mechanisms underlying some tumor development are correlated with germline and somatic mutations that involve the same cellular gene products. Two examples of the tumor suppressor genes are the Rb and p53 gene, which both

serve to act as a restraint to cell division in many tissues and their absence or inactivation is linked to the development of malignant tumors.¹⁵

Retinoblastoma gene (Rb gene) will cause a rare childhood cancer, retinoblastoma, when they are inactivated.¹⁵ Approximately 40% of the cases are associated with germline mutation, in which all somatic cells carry one missing or mutated allele of a gene and the remainders are not hereditary, requiring somatic mutations. The function of Rb genes is a crucial checkpoint in the cell cycle and inactivating them allows unregulated cell proliferation.¹⁷ They are also believed to be able to bind to numerous transcription factors either augment or antagonize their function and also to many other signaling molecules, indicating their regulatory functions.^{15,17}

The p53 tumor suppressor gene is the main mediator for apoptosis, growth arrest, and senescence. The level of p53 will rise in response to DNA damage, oncogenic activation of other proteins, and other stresses (e.g. hypoxia); thus, preventing the cells from progressing to the S phase, allowing time for DNA repair. The p53 protein is a transcriptional factor that promotes the expression of a number of other genes involved in the control of cell cycle and progression.

When DNA damage and other stresses occur, the upregulation of p53 will enhance the synthesis of CKIs (helped by CIP1), in which it will then inactivates cyclin/CDK complexes that leads to cell arrest at the G₁/S checkpoint. At this point, cells that are halted may undergo DNA reparation (helped by GADD45) and then reenter the cycle or undergo apoptosis.^{15,17}

Inactivating mutations of p53, which is mostly due to missense mutations that impairs the ability of p53 to bind with DNA, allows cells with damaged DNA to progress through the cell cycle.¹⁵⁻¹⁷ This gene is deleted or mutated in 75% of cases of various cancers further contributing the fact that mutations of p53 seem to be the most common genetic change in human cancer.¹⁶ Furthermore, the actions of mutant p53 molecules extend beyond inactivation of its tumor suppressing function. The anomalous protein also functions as oncogene modulating gene transcription; it actually protects cells from apoptosis. Mutant p53 also activates proinflammatory cytokines and extracellular matrix modulators. Moreover, these aberrant proteins can activate cellular mechanisms that are

responsible for resistance to chemotherapeutic drugs.¹⁵⁻¹⁷

2.3.10 DINOMIT Cancer Model

DINOMIT cancer model is a model consisting of the classical concept of carcinogenesis proposed by Garland et al. (2009).¹⁸ It consists of 7 stages of development, which includes Disjunction-Initiation-Natural selection-Overgrowth-Metastasis-Involution-Transition, hence the name DINOMIT.

The first phase is disjunction, where there is substantial weakening or loss of adherence between epithelial cells within a tissue compartment.^{18,19} Loss of E-cadherin synthesis is partly responsible and it is closely related to the insufficient concentration of beta-catenin at the plasma membrane to link the E-cadherins to the cytoskeleton tightly enough.¹⁸

It is then followed by the initiation phase. In this stage, there is a variation in the DNA or epigenetic factors that will influence the transcription and ultimately causing changes in the protein translation.¹⁵⁻¹⁸ The critical part is when there uncorrected errors that may persist, as explained previously that a persistent genetic or epigenetic variation is a condition required for cancer development.

Natural selection is the stage that occurs after disjunction of cells that allows mobility and some variation of DNA or epigenetic factors exists, creating a competitive population dynamic where selection of the fastest reproducing and most aggressive cells will prevail.^{18,19} Rapidly reproducing cells have no way of sensing their behavior may cause adverse, fatal, and even poses the risk of death of the organism. Thus this evolution-like condition is termed differently, and it is known as devolution.¹⁸ Since the driver of this devolution is the gene, a stem cell with genes that hold the ability for faster reproduction or aggression will overwhelm the other cells, thus over represented in the tissue compartment.

The event that follows is called overgrowth, where there is clonal expansion of the tumor outside the basement membrane of the tissue into the stromal layer.^{18,19} One possible reason for invasion is that the crowding of cells may starve them, and they will seek essential amino acids in the basement membrane. Aggressive cells near the basement membrane may start obtaining essential amino acids by various means, but almost always invading and

weakening the basement membrane, allowing further penetration. This leads to the overgrowth phenomenon in the stromal layer, which is clinically evident as localized cancer.^{18,19}

Metastasis is the next phase that can be regarded as colonization of a remote range. When expansion of tumor mass continues, a few cells may transit the lymphatic or blood circulation and ended up being lodged in remote tissues, where overgrowth continues if intercellular communication and differentiation are not restored.¹⁵⁻¹⁹ Disjunction of remote tissues may also provide a higher risk for the metastatic cell to easily invade the new location.¹⁸

The next phase in involution, which occurs when vitamin D status is restored by a seasonal rise in 25(OH)D; it consists of the temporary arrest of growth, metastasis, and other developmental process of primary tumor whose VDR is intact.^{18,19} During this phase, even though the malignant cells remain metastasized, their mitoses can be limited due to re-established intercellular junction if they still have intact VDR, thus no new malignant cells are produced.¹⁸ However if involution does not occur, then the tumor may expand and grow to a volume that is critical as it may hinder essential functions of vital organs.

The last phase is transition, which occurs if vitamin D and calcium deficiency persist and the metastatic lesions do not harm a vital organ, then these metastatic cancers will make a transition to disseminated malignancy.¹⁹ However it is important to remember that some malignancies are of nonepithelial origin, thus this model is not applicable to such cancers.¹⁸

2.4 The Correlation Between Vitamin D and Cancer

2.4.1 Cancer Prevention

Several levels are shown to support the association between vitamin D and cancer and this is also supported by *in vitro* and epidemiologic studies.¹⁰ Firstly, in low circulating levels of vitamin D, there is an increased risk of developing cancer. In contrast, a high intake of vitamin D is linked with a reduced risk of cancer.^{10,18,19} Furthermore, the aggressiveness of cancer seems to be lower during summer, when the production of vitamin D is higher.¹¹ Polymorphism of genes encoding proteins that are involved in the signaling pathway of vitamin D affects

the risk of cancer development. When tumor cells are exposed to high levels of vitamin D compounds, their proliferation are inhibited and differentiation is induced.^{10,18,19}

A study, which monitors the incidence of cancers throughout North America, shows difference in areas with different latitudes, suggesting that their subcutaneous production of vitamin D differs depending on their location in regards to sun exposure, thus affecting also the development of cancer in those varying individuals.¹⁹ Another study back in 1981 shows that 1,25(OH)2D3 in nanomolar concentrations was able to inhibit the proliferation of human melanoma cells substantially and stimulates the differentiation of myeloid leukemia cells.²⁰

Ten mechanisms have been identified in the role of vitamin D and calcium in reducing cancer incidence and mortality. These mechanisms include (1) up-regulation of adherence and signaling between epithelial cells; (2) contact inhibition of proliferation; (3) cell differentiation; (4) cell cycle stabilization; (5) apoptosis promotion; (6) anti-neoangiogenesis; (7) down-regulation of glycogen synthase kinase 3 (GSK-3), which will then reduce the proliferation of colorectal, prostate, and pancreatic cancers in vitro; (8) down-regulation of the canonical Wnt signaling pathway that is markedly active in colorectal cancer and other cancers as well; (9) increased expression of DKK-1 protein, a tumor suppressor in the colon cancer cells that has a mutation in Wnt/beta-catenin pathway; and (10) down-regulation of DKK-4 transcription (a target of the Wnt/beta-catenin pathway that is up-regulated in colorectal cancer that increases cellular autonomy, mobility, and invasiveness).^{18,19} The VDR-1,25(OH)2D complex will bind to the promoter of DKK-4 to prevent the transcription process in a large scale.

Vitamin D metabolites such as calcitriol can up-regulate the transcription of E-cadherins, the main epithelial intercellular adhesion proteins thus exerting the cancer preventing properties mentioned previously.^{18,19} Such occurrence enables translocation of beta-catenins into the plasma membrane, ultimately causing in anchoring of intercellular junctional proteins to the cytoskeleton to help maintain the normal shape of epithelial cells. A tight physical contact with adjacent cells within a tissue that strengthens the inhibition of proliferation is

termed as contact inhibition, and this can also be achieved only when the cells are closely attached to one and another.^{18,19} Cell-to-cell communication is also enhanced through gap junctions, which will further enhance the mutual adherence of cells.

Calcitriol also enhances the pulsatile release of ionized calcium from intracellular stores (i.e. endoplasmic reticulum), thus inducing terminal differentiation and apoptosis. The study conducted by Garland et al. (2009)¹⁸ regarding their proposed model of cancer pathogenesis, the DINOMIT model provides a better insight to the ability of vitamin D and calcium to prevent and potentially, arrest the pathogenesis of cancer.

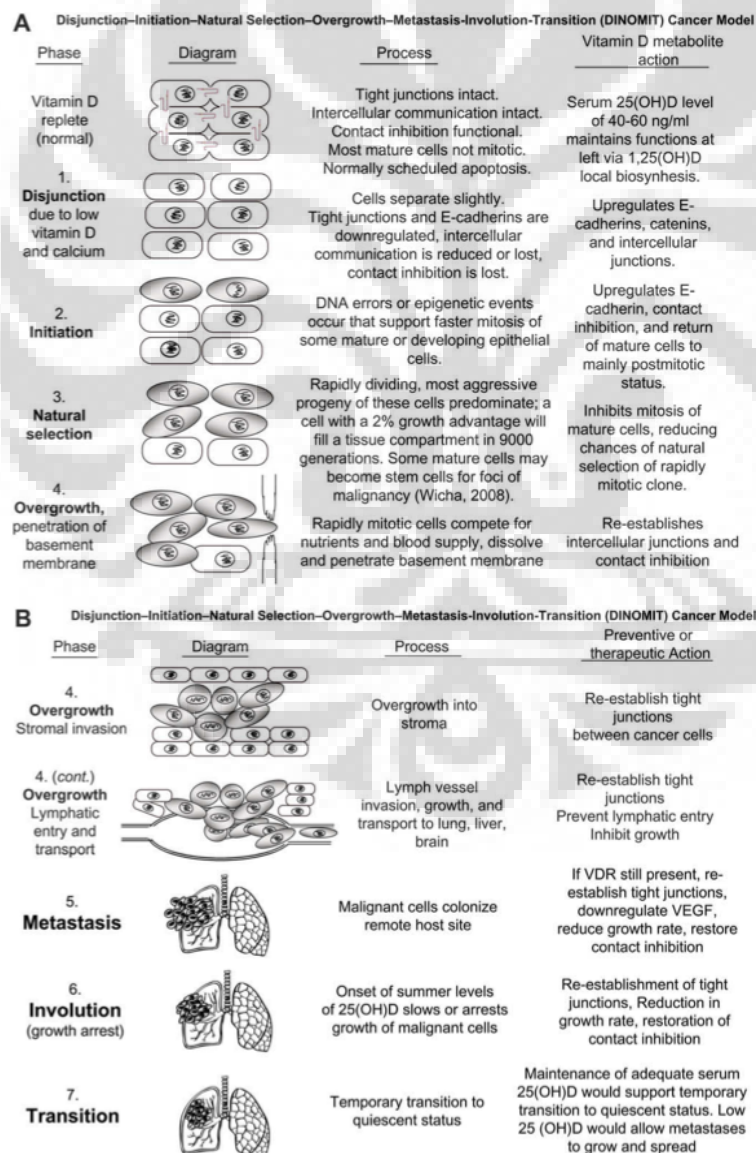


Figure 1 and 2.
DINOMIT Cancer Model (part A and B).
Taken from: Garland CF, Gorham ED, Mohr SB, Garland FC. Vitamin D for cancer prevention: global perspective. *Ann Epidemiol* 2009;19:468-483.

2.4.2 Implication Towards Vitamin D System

An up-regulation of VDR expression is seen in several tumors and is thought to represent an important endogenous response to the progression of tumor.¹⁰ A study conducted by Matusiak et al. (2005) shows that in normal colonic epithelial cells, the VDR levels were low, whereas in aberrant crypt foci, polyps, and well-differentiated tumor cells, an increased level of VDR is observed.²¹ However a decline followed the rise after said conditions, as de-differentiation that occurs due to the tumor cells function. This occurrence suggests that the progression of colon cancer may cause a reduced response to the action of vitamin D in comparison to normal condition, due to a reduced rate of this protein that translocates to the nucleus as the tumor progresses.¹⁰

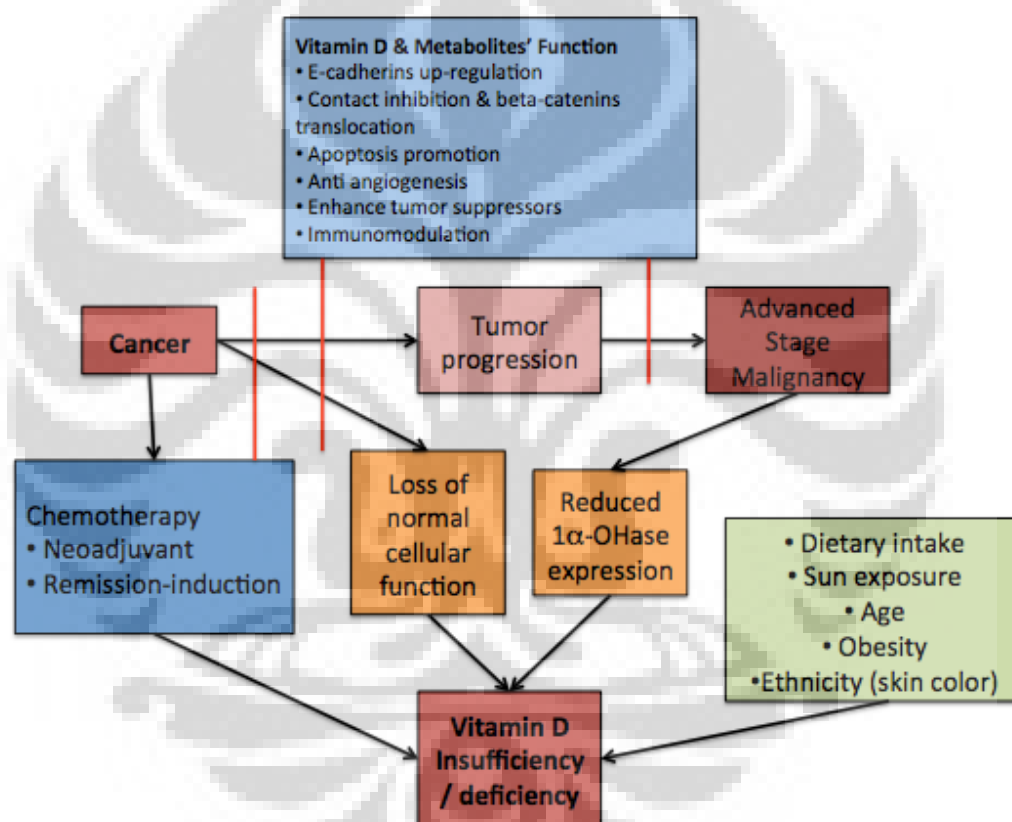
As mentioned previously, over-expression of VDR (which is seen in cholangiocarcinoma) seems to be an endogenous mechanism of response that is actually useful for mediating antiproliferative effects of calcitriol in these cell lines, thus leading to a better expected outcome as the desired effects are enhanced.^{16,18,19}

A reduced expression of 1α -OHase in malignant cells has been suggested but not uniformly observed in all tumor types.¹⁰ There is a high level of 1α -OHase expression in breast and colorectal cancer cells. Although there is a diversity observed regarding tumor types, this is expected to only cause a difference in the degree of protective factor of vitamin D against the onset and progression of cancer.^{15,18,19}

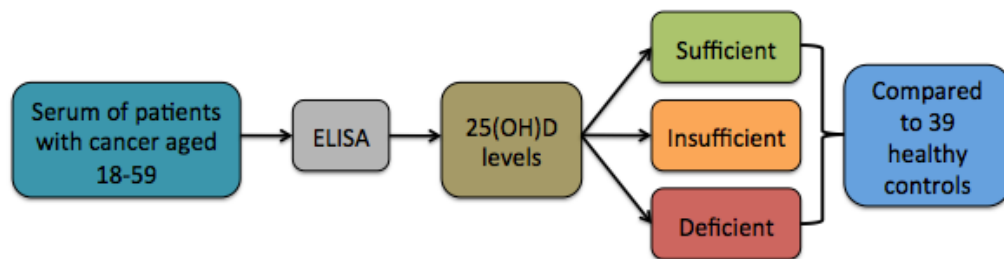
From points mentioned above, it is understood that cancer hinders the prognosis of a patient as it makes cells lose their function and reactivity towards vitamin D by reducing the number of VDR expression in progressed stages. The inhibition of 1α -OHase also hinders the transformation of 25(OH)D into $1,25(\text{OH})_2\text{D}_3$. Cancer may not directly cause a vitamin D insufficiency, however the treatment regiments such as chemotherapy does. A study conducted in France, 2011 compared the levels of 25(OH)D in locally advanced breast cancer patients before and after receiving neoadjuvant chemotherapy (NCT).²² Although 79.5% had already had baseline vitamin D insufficiency and 10.4% had the status of severe insufficiency prior to NCT, a marked increase was seen as 97.4% having

insufficiency and 23.4% having severe insufficiency.²² Another study published in 2013 from Pakistan, managed to support the notion of chemotherapy having effects to vitamin D levels as newly diagnosed patients of acute leukemia are evaluated from before and after their treatment using remission-induction chemotherapy.²³ Their results showed that over 90% of the patients they treated had insufficient 25(OH)D levels and it is significantly reduced after remission-induction chemotherapy as compared to the untreated group.²³

2.5 Theoretical Framework



2.6 Conceptual Framework



CHAPTER 3

METHODS

3.1 Research Design

The research design that is used for this study was comparing means between two independent populations. The comparison was done by measuring 25(OH)D levels between cancer patients and healthy controls, indicating that the samples came from two different groups, making it unpaired. The dependent variable in this study was the 25(OH)D levels, which were be measured in ng/mL, thus it is a numerical variable.

3.2 Time and Location of The Study

This research was done for 2 months, starting from May until June 2015. For this research, we used the facilities that are provided in the Department of Clinical Pathology, Rumah Sakit Cipto Mangunkusumo, Jakarta Pusat, Indonesia.

3.3 Data Sources

The data was from primary sources that were acquired from leftover serum collected in the Laboratory of Clinical Pathology Department, RSCM, Jakarta; in which the serum was then measured using immunoassay method.

3.4 Population and Sample

The population of this research were collected from cancer patients and serum of healthy controls, both from the Department of Clinical Pathology in Rumah Sakit Cipto Mangunkusumo, Jakarta.

The serums obtained are superfluous, leftover serums that were already taken previously by the laboratory for their previous examinations that were conducted under the agreement of the patient. All information regarding the patient's identity will not be disclosed under any circumstances.

3.4.1 Inclusion Criteria for Cancer Group

- Subjects with age ranging between 18-59 years old
- Subjects who have not received any treatment for cancer

- Subjects who have cancer, confirmed by crosschecking to a complete medical records with the following information:
 - White blood cell differential count
 - Tumor markers or diagnosis of physician

3.4.2 Exclusion Criteria for Cancer Group

- Subjects who have other underlying conditions aside from malignancies
- Subjects who lack the required information from the medical records

3.4.3 Inclusion Criteria for Healthy Controls

- Subjects with age ranging between 18-59 years old
- Subjects who are free from abnormalities in laboratory parameter

3.4.4 Exclusion Criteria for Healthy Controls

- Subjects who shows abnormalities in laboratory parameter
- Subjects receiving any kinds of treatment or medication

3.4.5 Dropout Criteria

- Contaminated samples

3.5 Sample Size

The sample for unpaired categorical-numeric analytic research will be calculated using a formula as follows:

$$n = 2 \left(\frac{(Z\alpha + Z\beta) S}{x_1 - x_2} \right)^2$$

In which,

n = total sample

Z α = standard deviation alpha (1.64)

Z β = standard deviation beta (1.28)

S² = combined standard deviation (obtained from previous similar research in Indonesia)

$X_1 - X_2$ = minimum significant difference in mean (difference between mean level of 25(OH)D in healthy controls and mean of serum level of vitamin D in cancer patients obtained from previous similar research done by Imtiaz S, Siddiqui N, Raza SA, Muhammad A from Shaikh Zayed Medical Complex, Lahore, Pakistan in 2012 titled “Vitamin D deficiency in newly diagnosed breast cancer patients”.²⁴ Thus $X_1 - X_2 = 14.9 \text{ ng/ml} - 9.3 \text{ ng/ml}$)

In order to determine the combined standard deviation (S^2), the difference of value between 25(OH)D levels between cancer patients and healthy controls from previous research in Pakistan is used. It is a research done back in 2012 by Imtiaz S, Siddiqui N, Raza SA, Muhammad A from Shaikh Zayed Medical Complex, Lahore, Pakistan in 2012 titled “Vitamin D deficiency in newly diagnosed breast cancer patients”²⁴ in which from 90 breast cancer patients, the serum level of vitamin D was $9.3 \pm 4.7 \text{ ng/ml}$, while from 90 healthy controls, the serum vitamin D level was $14.9 \pm 10.3 \text{ ng/ml}$.

Thus, to determine the combined standard deviation (S^2), the formula below is used:

$$S^2 = \frac{(s_1^2 \times (n_1 - 1) + s_2^2 \times (n_2 - 1))}{n_1 + n_2 - 2}$$

In which,

S^2 = combined standard deviation

s_1 = standard deviation of cancer patients group from previous research (4.7 ng/ml)

n_1 = sample size of cancer patients from previous research (90)

s_2 = standard deviation of healthy controls from previous research (10.3 ng/ml)

n_2 = sample size of healthy controls from previous research (90)

Thus, the calculation is

$$S^2 = \frac{(4.7^2 \times (90 - 1) + 10.3^2 \times (90 - 1))}{90 + 90 - 2}$$

$$S^2 = \frac{(1966.01 + 9442.01)}{178}$$

$$S^2 = 64.09$$

As a result, the calculation of sample size is as follows:

$$n = \frac{2(Z_{\alpha} + Z_{\beta})^2 \times S^2}{X_1 - X_2^2}$$

$$n = \frac{2(1.64 + 1.28)^2 \times 64.09}{(14.9 - 9.3)^2}$$

$$n = \frac{2(2.92)^2 \times 228.83}{5.6^2}$$

$$n = 34.8505$$

According to the equation, the sample size needed is 34.85; and it will be rounded to 35.

3.6 Research Methods

3.6.1 Variable Identification

- Independent:
 - Serum of patients with cancer
- Dependent:
 - The level of 25(OH)D in the serum, measured in ng/mL

3.6.2 Materials & Equipment

In this study, total vitamin D from the serum of patients are measured using total 25-Hydroxyvitamin D reagent kit (ref no.

05894913190) from Roche and processed with cobas® e 411 immunoassay analyzer. The reagents used were as follows:

- Pretreatment reagent 1 (PT1) → Dithiothreitol 1 g/L, pH 5.5
- Pretreatment reagent 2 (PT2) → Sodium hydroxide 55 g/L
- Streptavidin-coated microparticles (M) → Streptavidin-coated microparticles 0.72mg/mL, preservative
- Vitamin D binding protein-BPRu (R1) → Ruthenium labeled vitamin D binding protein 150 µg/L, bis-tris propane buffer 200 mmol/L, albumin (human) 25 g/L, pH 7.5, preservative
- 25-hydroxyvitamin D-biotin (R2) → Biotinylated vitamin D (25-OH) 14 µg/L, bis-tris propane buffer 200 mmol/L, pH 8.6, preservative

However, there are some additional materials that are required for calibration purposes, which are:

- Vitamin D total Calset (ref no. 05894921190)
- Precicontrol Varia (ref no. 05618860190)

3.6.3 Preparation

3.6.3.1 Equipment Calibration

Calibration is done prior to the use of immunoassay analyzer using a 2-point calibration.

3.6.3.2 Sample Preparation

The serum specimens were collected and labeled respectively. Centrifugation for 5 minutes was done only for blood samples, in which then the serum may be obtained. A minimal amount of 0.1mL is needed for each sample.

3.6.4 Process of Data Collection

The samples were loaded into the holder of the cobas ® e 411 immunoassay prior to starting the machine. The serum 25(OH)D levels were expressed in terms of ng/mL and the processing went for 27 minutes. The principle of the test includes:

- 1st incubation: The sample is incubated with pretreatment reagent 1 and 2, releasing vitamin D (25-OH) from the vitamin D binding protein.
- 2nd incubation: The pretreated sample is then incubated with ruthenium labeled vitamin D binding protein, forming a complex between the vitamin D (25-OH) and the ruthenylated vitamin D binding protein.
- 3rd incubation: Streptavidin-coated microparticles and vitamin D (25-OH) labeled with biotin is added, which occupied the unbound ruthenium labeled vitamin D binding proteins. A complex consisting of the ruthenylated vitamin D binding protein and the biotinylated vitamin D (25-OH) is formed and becomes bound to the solid phase.
- After the third incubation the mixture is then aspirated into the measuring cell and the microparticles are captured onto the surface of the electrode magnetically. Unbound substances are removed using ProCell/ProCell M.
- Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.
- The results are determined by comparing with the calibration curve generated and a master curve provided by the reagent kit.

3.6.5 Operational Definition

- Serum of healthy controls: the serum of subjects with all laboratory parameters within the normal limits
- Serum of cancer patients: the serum of subjects with signs of malignancy confirmed using the medical records by means of their diagnosis or specific markers
- 25(OH)D: 25-hydroxyvitamin D, a form of vitamin D that is used to measure and represent the total vitamin D of the patient

- Immunoassay: a biochemical test that works to measure the presence of a macromolecule through the use of an antibody
- Vitamin D deficiency: when 25(OH)D level is <10.00 ng/mL
- Vitamin D insufficiency: when 25(OH)D level ranges between $10.00 - 29.99$
- Vitamin D sufficiency: when 25(OH)D level is >29.99 ng/mL

3.7 Data Analysis

3.7.1 Statistical Analysis

Medical records of each subject is used to acquire the gender, age, and differential count, specifically lymphocyte count, in order to supplement their data on 25(OH)D levels.

Independent T-test is used to compare and analyze the significance of the mean 25(OH)D levels between the cancer patients and healthy controls.

A correlation between 25(OH)D levels and lymphocyte count is also analyzed, where the 25(OH)D levels are categorized. The means from both variables were compared using One-way ANOVA test.

The gender of the subjects are also analyzed for the comparison and significance with the mean 25(OH)D levels using Independent T-Test.

Lastly, age is analyzed to look for their correlation, in which they are categorized per decade to be analyzed with the means of 25(OH)D level in each age group and compared using One-way ANOVA test.

CHAPTER 4

RESULT

This study used 35 serum obtained from cancer patients and 39 from healthy controls, and they were processed using the cobas® e 411 immunoassay analyzer in order to read the the serum 25(OH)D levels. All of the data is are then processed further using IBM® SPSS® Statistics Version 20. The mean serum vitamin D levels between the two groups are illustrated in the following graph.

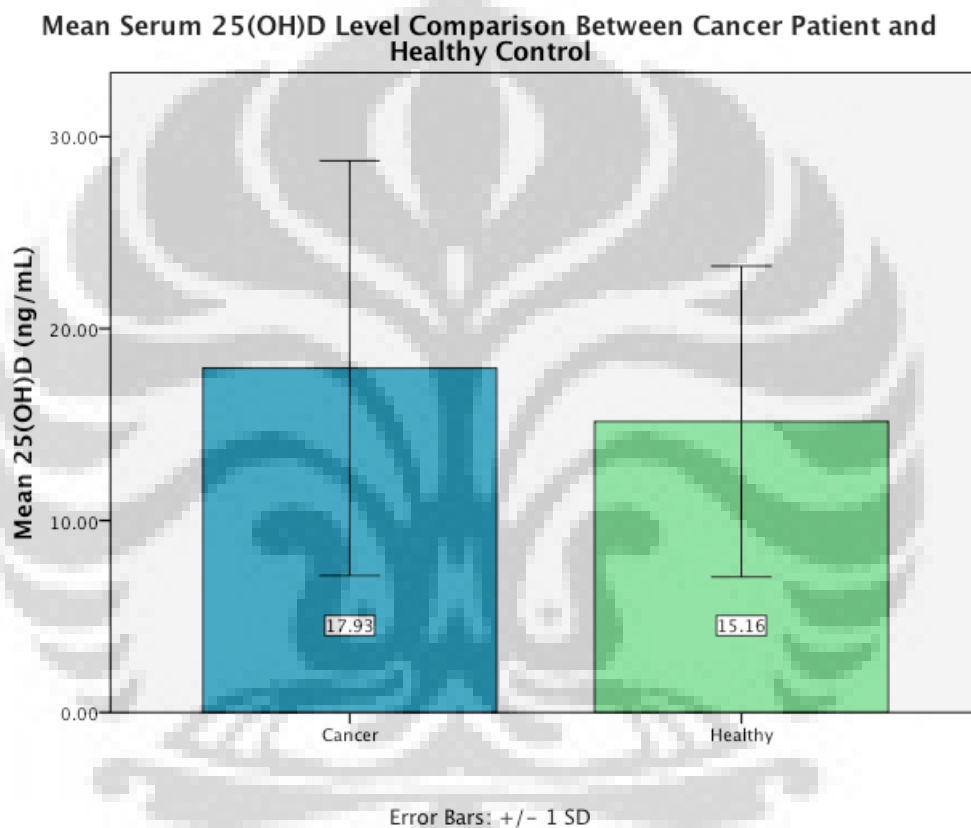


Figure 3. Mean Serum 25(OH)D Level of Cancer Patients in Comparison to Healthy Controls

The graph shows that the mean serum level of cancer patients is slightly higher than the healthy controls, where the former is at 17.93 ± 10.81 ng/mL and the latter is at 15.16 ± 8.10 ng/mL. The highest serum vitamin D level in the cancer patients group is at 40.89 ng/mL, whereas it is at 42.69 ng/mL in the healthy controls group.

As the total number of samples exceeded 50 (Appendix 2 Table 4), then Kolmogorov-Smirnov is used for the normality test. However, the data

distribution found indicates that it is not normal, thus the data is transformed using Lg10 and the same test is conducted again, finally showing a normal data distribution (Appendix 2 Table 5) with $p > 0.05$. Further analysis is done using Independent Sample T-test (Appendix 2 Table 6). The Levene's test for equality of variances shows that $p < 0.05$ thus equal variances are not assumed. The value for equal variances not assumed is .671, which indicates that $p > 0.05$ ($p > 0.05$, $p = 0.671$) thus the data analysis through Independent Sample T-test is insignificant.

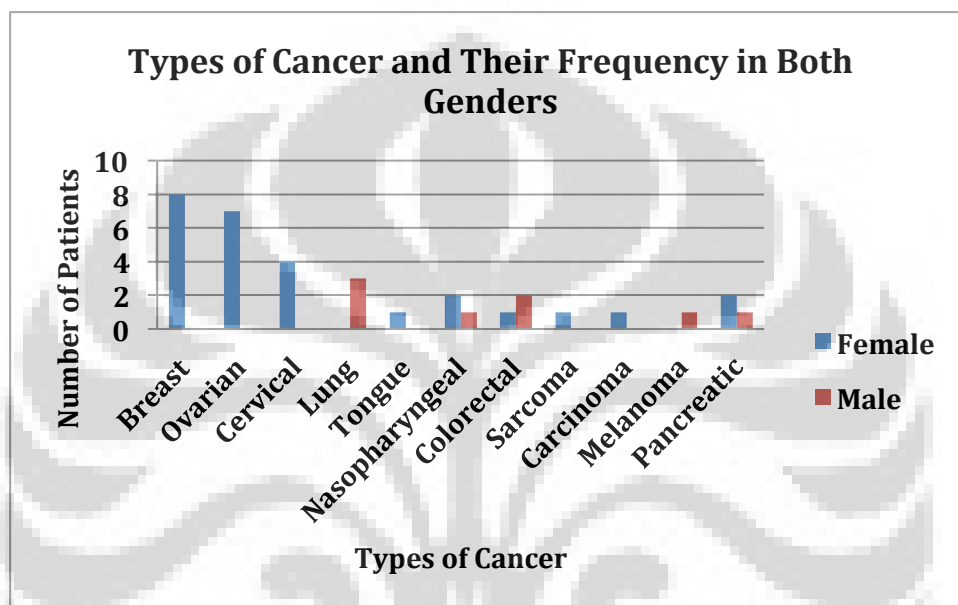


Figure 4. Distribution of the Types of Cancer in Cancer Patients

The graph above shows the distribution among the cancer patients included in this study, in which they are also divided by gender. It is indicated that number of breast cancer patients in female is the highest (8 patients out of 27 female patients). As for the male cancer patients, the highest are shown to have lung cancer (3 patients out of 8 male patients).

A pattern of correlation between the 25(OH)D levels and the lymphocyte count was also sought after, with the patients' lymphocyte count is known from the white blood cell differential count obtained from the medical records. The graph below shows the results from the search of said cause.

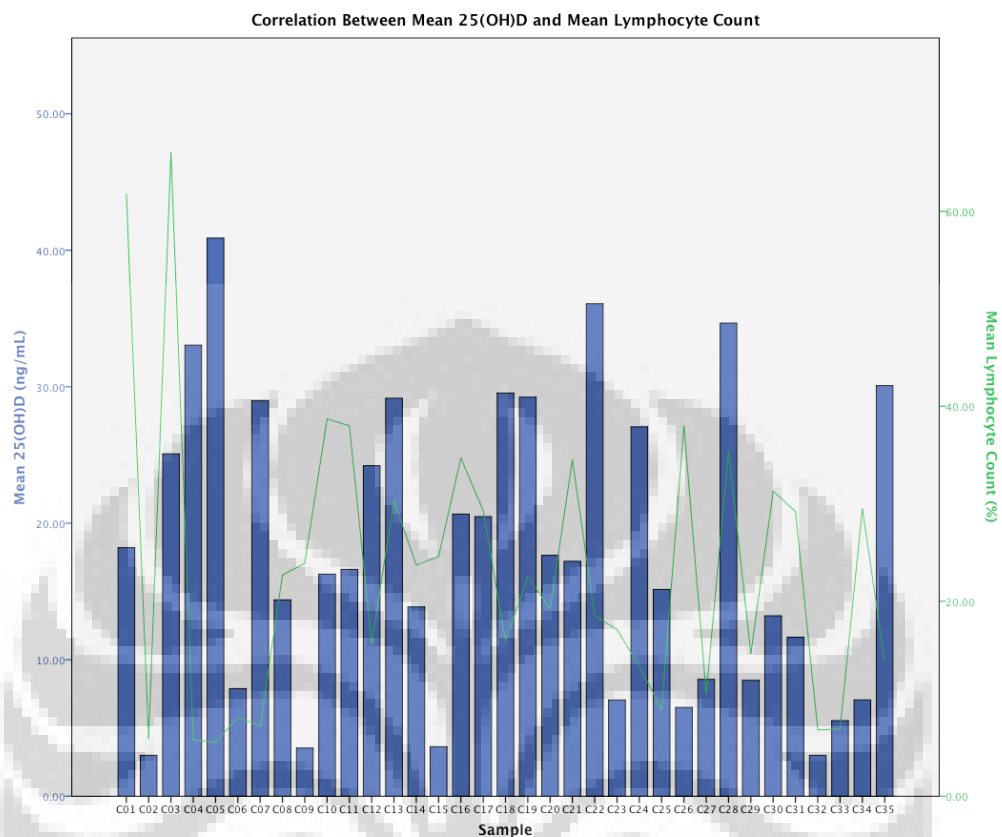


Figure 5. Comparison Between the Values of 25(OH)D and Lymphocyte Count

There are no noticeable pattern of correlation between 25(OH)D levels and the lymphocyte count, indicating that there is no correlation of any significance from comparing the two.

As the samples used in this case is from the 35 patients with cancer, which is less than 50, thus Shapiro-Wilk is the test of choice for normality (Appendix 3 Table 8). The p values for both 25(OH)D and lymphocyte count are $p > 0.05$ and $p < 0.05$ respectively, indicating that the data distribution for the lymphocyte count is not normal. A transformation using $Lg10$ is done again for the lymphocyte count data (Appendix 3 Table 9). Prior into proceeding further with the analysis using both data, a scatter/dot graph was made to check for a pattern of linearity (Appendix 3 Figure 9). From the results of the graph, the data is seen to be scattered with no pattern of linearity, thus an analysis for correlation is not favorable in this condition, and a comparative analysis serves a better purpose.

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The test used was One-way ANOVA test using lymphocyte count as the dependent variable and a category for vitamin D as the factor, which includes (1) Deficiency: <10.00 ng/mL, (2) Insufficiency: $10.01 - 29.99$ ng/mL, (3) Sufficiency: >29.99 ng/mL. From the initial normality test using Shapiro-Wilk (Appendix 3 Table 11) the data distribution is not normal as the $p < 0.05$, thus a transformation using $\text{Lg}10$ was done, producing a normal data distribution with $p > 0.05$ (Appendix 3 Table 12).

A test of homogeneity of variances was done for the One-way ANOVA test and it shows that $p > 0.05$ (Appendix 3 Table 13), thus there are equal variances. However, the result of the ANOVA shows that the p value is .095, which is greater than 0.05 ($p > 0.05$, $p = 0.095$), therefore there are no significant mean differences between the groups (Appendix 3 Table 14).

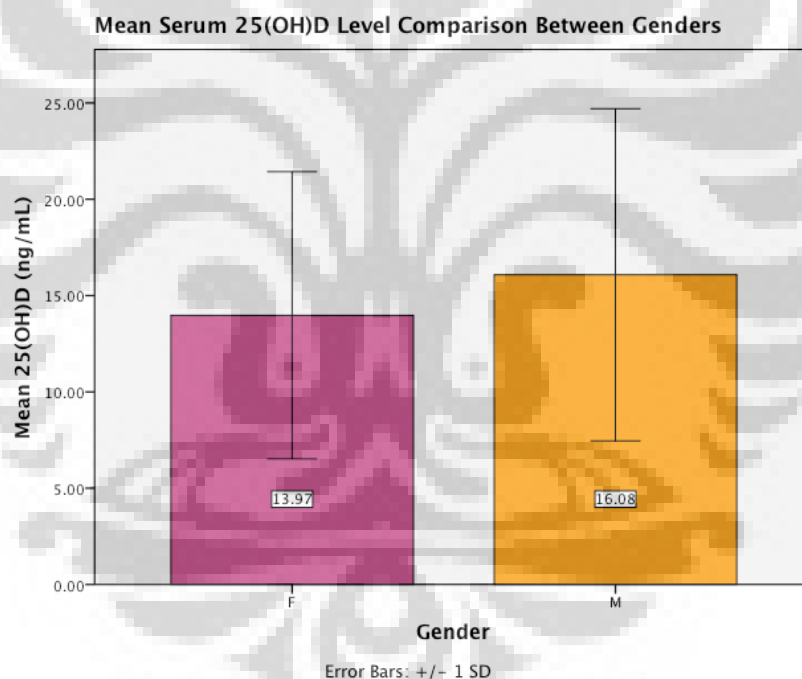


Figure 6. Mean Serum 25(OH)D Level Comparison Between Genders in Healthy Controls

The graph for the total population shows that the mean is higher in the male population as opposed to the female population at 16.08 ± 8.62 ng/mL and 13.97 ± 7.46 ng/mL respectively. The highest level of 25(OH)D in male recorded is 42.69 ng/mL, whereas it is 27.73 ng/mL in female. In addition, the lowest level of serum vitamin D level in male is 5.93 ng/mL and 4.19 ng/mL in female.

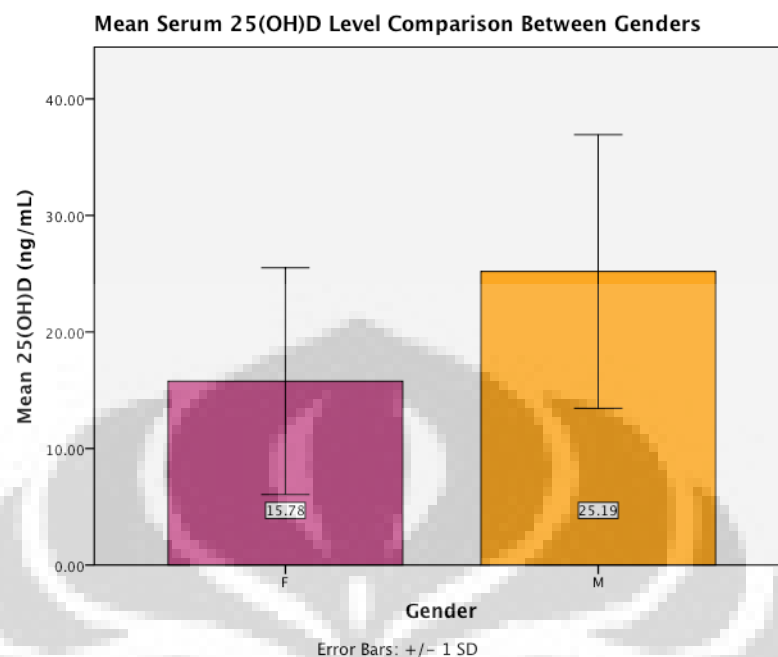


Figure 7. Mean Serum 25(OH)D Level Comparison Between Genders in Cancer Patients

For the graph in cancer patients, one fact remains the same and that is the male group has the higher mean 25(OH)D level compared to the female group. The mean level for the former group is at 25.19 ± 11.74 ng/mL, whereas the latter is at 15.78 ± 9.73 ng/mL. There is approximately 10.00 ng/mL difference between the mean 25(OH)D level between the male population and the male cancer patients group. Secondly, the mean of the female cancer patients group is approximately 3.00 ng/mL lower than mean of the total female population.

Statistical analysis is done only in the cancer patients group, and a normality test using Shapiro-Wilk shows a normal data distribution with $p > 0.05$ (Appendix 4 Table 16), therefore it is eligible to be used directly to an Independent Sample T-test as there are only two groups of gender. Equality of variance is known from Levene's test and its result shows that $p > 0.05$, thus equal variances are assumed (Appendix 4 Table 17). The p value for equal variances is .028 thus it is $p < 0.05$, which indicates that the result for this Independent Sample T-test is significant ($p < 0.05$, $p = 0.028$).

The last but not least is the analysis for 25(OH)D levels in each age group that has been categorized per decade that is well within the range of age accepted in this study. Two graphs were also created, a graph that includes only the cancer patients group and another that combines all population in this study for a comparison between the two as follows.

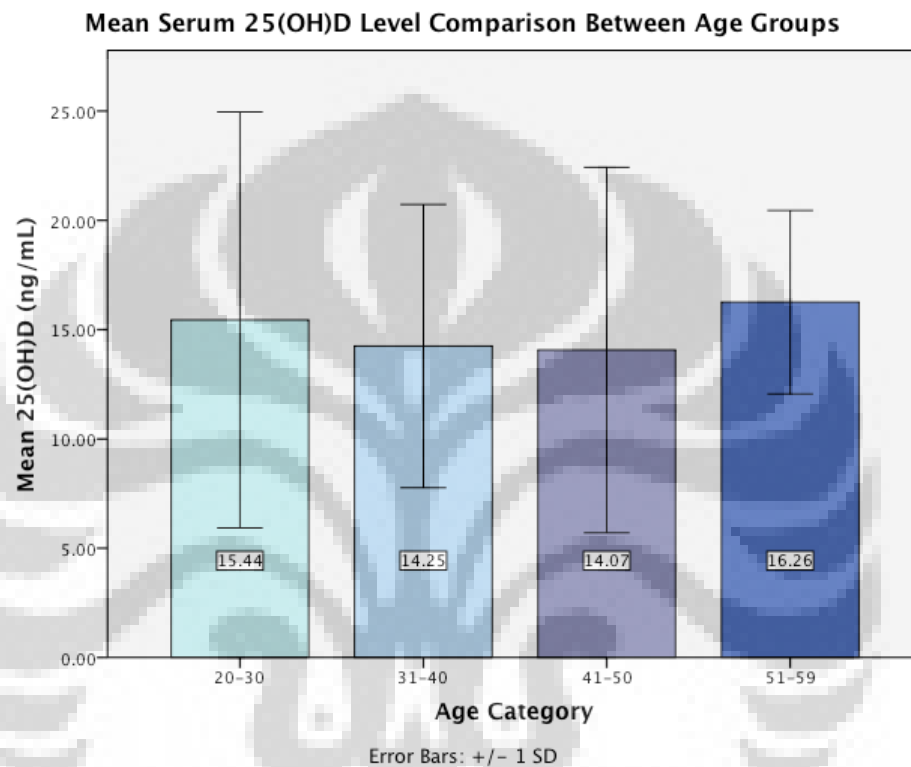


Figure 8. Mean Serum 25(OH)D Level Comparison Between Each Age Groups in Healthy Controls

The graph for the total population does not pose a certain pattern in general for their respective means (20-30: 15.44 ± 9.51 ng/mL, 31-40: 14.25 ± 6.48 ng/mL, 41-50: 14.07 ± 8.34 ng/mL, 51-59: 16.26 ± 4.19 ng/mL).

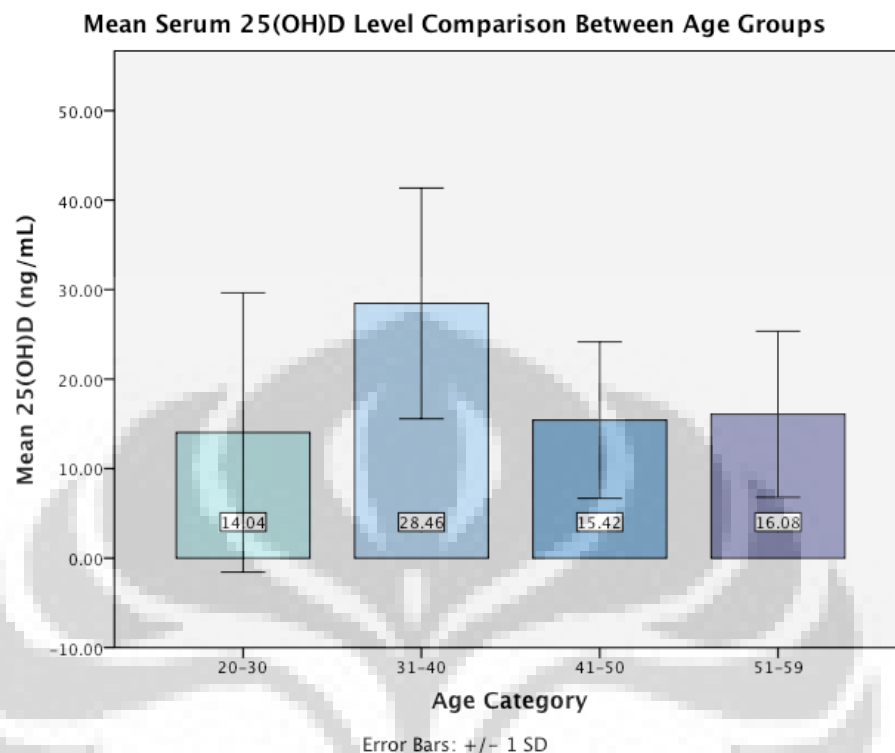


Figure 9. Mean Serum 25(OH)D Level Comparison Between Each Age Groups in Cancer Patients

A similar data distribution can be seen in general for this group for their respective means in each of the age groups (20-30: 14.04 ± 15.61 ng/mL, 31-40: 28.46 ± 12.90 ng/mL, 41-50: 15.42 ± 8.73 ng/mL, 51-59: 16.08 ± 9.28 ng/mL). Overall, there are no significant difference of mean between most of cancer patients and healthy controls, however the highest mean 25(OH)D levels in the cancer patients and healthy controls differs, in which it is highest in the 51-59 age group in the healthy controls group, whereas it is highest in the 31-40 age group in the cancer patients group.

Just like what was done in gender comparison, only the cancer patients group was analyzed further using One-way ANOVA test. Prior to the test mentioned, a normality test was conducted using Shapiro-Wilk and the p value is >0.05 (Appendix 5 Table 19), indicating a normal data distribution. The age group is categorized in the same manner as in the graphs. Homogeneity of variances is tested for the One-way ANOVA test and it shows that $p > 0.05$ (Appendix 5 Table

20), therefore there are equal variances. The result of the ANOVA shows that the p value is .066, greater than 0.05, indicating that there are no significant mean differences between the groups ($p > 0.05$, $p = 0.066$).



CHAPTER 5

DISCUSSION

From the previous chapter, there is only a slight difference between the mean serum 25(OH)D levels in cancer patients and healthy controls, 17.93 ± 10.81 ng/mL and 15.16 ± 8.10 ng/mL respectively, where the former possess a slightly higher number and statistically the p value is >0.05 ($p = 0.427$), showing no significant difference after it was tested using Independent Sample T-test. This data then opposes the hypothesis, proving that the mean serum 25(OH)D level of cancer patients, is in fact, higher than the healthy controls.

It is ironic, knowing the fact that Indonesia is a tropical country, which has an abundant sunlight, has a mean of 15.16 ± 8.10 ng/mL in serum vitamin D level from the group that is supposed to represent the general population that is assumed, healthy. Out of 39 healthy controls, only about 2.56% of the healthy controls that can be categorized into sufficiency and approximately 53.85% is classified into having vitamin D deficiency, whereas the estimated remaining 43.59% is considered vitamin D insufficient. When taking gender into account within the healthy controls, the mean serum 25(OH)D level for women (15.08 ± 8.87 ng/mL) is lower than the level in men (18.51 ± 10.19 ng/mL). The same condition occurs in the group of cancer patients where female is lower than the male, at 15.78 ± 9.73 ng/mL and 25.19 ± 11.74 ng/mL respectively with a $p < 0.05$ ($p = 0.028$), indicating that there is a significant difference between the two. Another factor that was taken into consideration is age, which data has already been shown in Chapter 4. Overall, from the data that are collected, the vitamin D profile in Jakarta, Indonesia may be said to be under the sufficient values.

There are several factors that may support the reasoning behind these data to be the way they are now. As for gender, men are more likely to be exposed to occupational reasons compared to women; furthermore women tend to stay away from the sunlight for cosmetic reasons. The effects from lack of exposure are then amplified by skin color, as darker skin color means more melanin content, creating a competitive relationship with 7-dehydrocholesterol, a precursor of vitamin D, for an essential factor in vitamin D production, UVB. Ethnicity in Indonesia may provide rough information on their skin color, which relates to

their melanin level but such information is not available from the medical records, providing a limitation of this study. Age is also a contributing factor to vitamin D deficiency, as the number of 7-dehydrocholesterol in skin will decrease as a person ages. Other factor that actually plays a role towards the vitamin D balance in the body is body fat, the relationship between body fat content and the amount of vitamin D that is sequestered in body fat is in direct proportion, which means that the higher body fat content a person has, then the higher amount of vitamin D that will be sequestered. Body fat may be known through BMI, however the medical records lack the information regarding this matter thus adding the limitations faced in this study. Last but not least, occupation may also be a factor because it is related to the exposure time to sunlight. It is safe to assume that most people in a metropolitan city like Jakarta have occupations indoors, which limits their exposure to sunlight and occupational data is also not available in the medical records thus estimating exposure of the subjects towards sunlight cannot be done.

Comparing the figures that are produced from this study to a previous one cannot be done, as there are no existing studies regarding the vitamin D profile of adult population in Indonesia, let alone Jakarta. A study in Singapore, a geographically similar country to Indonesia, that was conducted by Robien et al. in 2013 showed that the mean serum 25(OH)D level was 27.4 ± 7.3 ng/mL.²⁵ A similar value with the mean 25(OH)D level obtained in this study (15.16 ± 8.10 ng/mL) is seen in Pakistan, another Asian country, in a study conducted by Imtiaz et al. in 2012, showed the mean serum of 25(OH)D level was 14.9 ± 10.3 ng/mL. Factors that may cause this variety or similarity are generally the same as the ones mentioned previously, which mostly involves behavior, sunlight exposure, ethnicity, and lifestyle.

Several other studies have shown how vitamin D deficiency increases the risk of developing cancer and vice versa. In this study however, the mean serum level in cancer patients is actually higher than the healthy controls (17.93 ± 10.81 ng/mL vs. 15.16 ± 8.10 ng/mL), which signifies that cancer patients in Jakarta are considered vitamin D insufficient, even though the difference is statistically insignificant ($p = 0.427$). Cancer treatment regimens such as chemotherapy

contribute to causing vitamin D deficiency, and even the cancer itself may not affect 25(OH)D level directly as it only inhibits the 1α -OHase, affecting the conversion of 25(OH)D into $1,25(\text{OH})_2\text{D}_3$, however it is marked in several types of cancer shows over-expression of VDR, which may consume more active forms of vitamin D, increasing the conversion from 25(OH)D, thus decreasing the circulating 25(OH)D level. Progression of cancer also affects the vitamin D level in the body, where in advanced stages 1α -OHase, VDR level, and reactivity towards the vitamin D system is decreased, causing a deficiency of vitamin D as an end result. However, the samples that are collected from the cancer patients are the ones that have not received any form of treatment for their cancer, meaning that it is naïve, thus eliminating a risk factor for the decrease in vitamin D levels. Furthermore, the data regarding the cancer staging is not available in the medical records, making it another limitation that this study faced. Jacot, Pouderoux, Thezenas et al. (France, 2011) studied and compared the levels of 25(OH)D in locally advanced breast cancer patients prior to neoadjuvant chemotherapy and post-intervention, indicating that 97.4% having insufficiency and 23.4% having severe insufficiency after the treatment, from 79.5%, which had vitamin D insufficiency and 10.4% that had the status of severe insufficiency.²² Another study conducted by Naz, Qureshi, Shamsi, and Mahboob T (Pakistan, 2013) supported this correlation as an evaluation before and after remission-induction chemotherapy is done to newly diagnosed patients of acute leukemia, where 90% of the patients had vitamin D insufficiency and their 25(OH)D levels were significantly reduced after the therapy ($p < 0.05$).²³ As for cancer progression, the study conducted by Imtiaz et al. (Pakistan, 2012) showed an association of mean serum vitamin D level with stages of breast cancer (Stage I: 12.75 ± 5.76 ng/mL, Stage II: 9.18 ± 5.31 ng/mL, Stage III: 8.49 ± 3.18 ng/mL).²⁴

The distribution of cancer types among the patients included in this study may indirectly represent the high number of breast cancer cases here in Indonesia. As the graph shows, the highest occurrence is breast cancer in female, followed closely by ovarian cancer, and cervical cancer is lower than both of them. This is supported by the fact that. As for the male cancer patients, lung cancer has the highest occurrence. A factor that may influence the distribution of lung cancer is

predominated by male patients is that amount male population in Indonesia that smokes is considerably more compared to the female population, as this increases the risk of smokers to develop lung cancer. Previous study done by Tjindarbumi and Mangunkusumo (Indonesia, 2002) showed that in the late 90s, approximately 12.10% cancer patients have breast cancer (ranked 2nd), 19.18% have cervical cancer (ranked 1st) and 5.31 have ovarian cancer (ranked 5th) in Indonesia, and they are included in the 10 most frequent primary cancer in Indonesia.²⁶ These three types of cancer, that is also shown to have a high frequency in this study, is included in the 10 most frequent primary cancers in females as well, in which it accounts around 17.77% for breast cancer (ranked 2nd), 28.66% for cervical cancer (ranked 1st), and 7.77% for ovarian cancer (ranked 3rd).²⁶ As for the 10 most frequent primary cancer in males, lung cancer accounts for 3.99% (ranked 10th). Nasopharyngeal cancer is ranked 2nd at 11.27% in males, whereas rectal cancer is ranked 5th, and colon cancer is ranked 9th at 7.07% and 4.17% respectively.²⁶ In a different study conducted by Youlden, Cramb, Yip, and Baade (Australia, 2014) it is mentioned that in 2012, it is estimated that in Indonesia there was 48,998 breast cancer incidence with an incidence rate of 12%, making Indonesia one of the highest number of breast cancer diagnosed within the Asia-Pacific region.²⁷

This study also explored and analyzed the possibility of a relation between serum 25(OH)D levels and lymphocyte count. As mentioned previously in Chapter 4, a correlative test is not suitable in this study due to the data distribution and One-way ANOVA test was done instead. Unfortunately, the result indicates that $p > 0.05$ as the p value is 0.095, which indicates the lack of significance in the mean differences between the groups (Appendix 3 Table 14). The data for vitamin D was grouped into three categories, which are (1) Deficiency: <10.00 that accounts for roughly 57.14% of the cancer patients, (2) Insufficiency: $10.01 - 29.99$ that accounts for approximately 37.14% of the cancer patients, and (3) Sufficiency: >29.99 that accounts for the estimated remaining of merely 5.71% of the cancer patients. Such distribution may be the cause of the insignificance in the test, as only a very small portion exists in one of the category (Appendix 3 Table 12), causing a limitation for this study. Comparison to previous studies in this

matter cannot be done as there are none that specifically links 25(OH)D to the lymphocyte count of the subjects and statistically proven. In spite of that, studies conducted by Hewison in 2010 and 2012 elaborates that vitamin D has effects experimentally on the immune system, such as influencing T lymphocyte responses by T-helper 1 lymphocytes, T-cell proliferation, T-helper 1 cells regulation, and T-helper 2 cells response.^{6,7} Due to the limitation encountered regarding this matter, the results are indeterminate.

The predominant constraint this study comes up against is the samples, as they are superfluous serums that are used previously in the laboratory for various examinations and the medical records can only be as thorough as formerly needed. Some of them were addressed previously for the healthy control, such as ethnicity for skin color, height and weight for BMI, occupation for sunlight exposure that are not available in the medical records, restricting the exploration of possiblites in regards to factors affecting vitamin D level. Moreover, the samples for the cancer group are mostly collected from a pre-operative situation, thus lacking the information regarding the stages of cancer at the time, ensuring an untreated cancer in every way but confining a risk factor for the vitamin D level of the cancer patients.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

1. There is no noteworthy difference of serum vitamin D level between patients with cancer and healthy controls, in which it is slightly higher in cancer patients compared to the healthy controls.
2. There is no significant relation between lymphocyte count and serum vitamin D level in cancer patients.
3. Cancer patients in Rumah Sakit Cipto Mangunkusumo, Jakarta, are mostly considered having a deficiency in vitamin D.

6.2 Recommendation

1. A similar study but using more primary data and serum that are obtained specifically for this purpose, in which many additional information required can be included by means of a comprehensive history taking or substantial questionnaire in order to reduce the amount of limitations.
2. A study that explores the possible relations between 25(OH)D levels and absolute lymphocyte count that is analyzed statistically.
3. A long-term study that involves vitamin D supplementation to cancer patients in order to gain a more in-depth understanding of their efficacy in projecting a better prognosis of such patients and their role in preventing the disease progression.
4. A study to investigate the cancer preventive abilities of vitamin D may be conducted by giving supplementation to a group of high-risk individuals and compare them to the control group.

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APPENDICES

APPENDIX 1

Table 1. Data Recording of Healthy Controls

Sample	Age	Gender	25(OH)D (ng/mL)
N1	24	F	8.74
N2	24	F	23.63
N3	26	M	10.57
N4	29	F	24.35
N5	29	F	27.73
N6	38	M	18.00
N7	31	M	9.41
N8	25	M	8.23
N9	26	M	22.59
N10	21	F	24.71
N11	32	M	11.69
N12	30	M	11.71
N13	47	M	25.77
N14	32	F	16.25
N15	47	M	5.93
N16	49	F	12.51
N17	51	M	13.68
N18	28	M	42.69
N19	32	F	13.63
N20	54	M	20.63
N21	32	F	6.32
N22	28	F	7.33
N23	23	M	13.38
N24	34	M	11.26
N25	26	M	10.41
N26	27	M	10.45
N27	24	F	6.09
N28	29	F	15.77
N29	30	M	24.69
N30	25	F	8.85
N31	26	M	7.54
N32	33	M	27.43
N33	25	F	17.90
N34	48	M	12.06
N35	51	F	11.76
N36	52	M	20.83
N37	57	M	14.39
N38	21	F	7.76

N39	27	F	4.19
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Table 2. Data Recording of Cancer Patients

Sample	Age	Gender	25(OH)D (ng/mL)	Lymphocyte Count	Type
C1	49	F	18.21	61.8	Breast
C2	52	F	3.00	5.9	Ovarian
C3	26	F	25.08	66.0	Cervical
C4	35	M	33.05	5.8	Lung
C5	34	M	40.89	5.5	Lung
C6	59	F	7.89	8.1	Breast
C7	38	F	38.97	7.2	Tongue
C8	51	F	14.38	22.7	NPC*
C9	44	F	3.53	23.9	Breast
C10	56	F	16.27	38.7	Ovarian
C11	58	F	16.62	38.0	Ovarian
C12	55	M	24.22	15.7	Colorectal ^a
C13	52	M	29.16	30.3	NPC*
C14	58	M	13.87	23.7	Lung
C15	40	F	3.63	24.6	Sarcoma
C16	48	F	20.67	34.7	Breast
C17	42	F	20.49	29.3	Colorectal ^b
C18	35	F	29.54	15.9	Ovarian
C19	54	F	29.24	22.5	Ovarian
C20	59	F	17.65	19.2	Breast
C21	56	M	17.21	34.5	Colorectal ^b
C22	56	M	36.08	18.5	Melanoma
C23	58	M	7.05	17.1	Pancreatic
C24	57	F	27.06	13.3	Pancreatic
C25	55	F	15.15	8.9	Ovarian
C26	55	F	6.51	38.0	Breast
C27	53	F	8.56	10.5	Cervical
C28	37	F	34.67	35.4	NPC*
C29	56	F	8.49	14.6	Carcinoma
C30	47	F	13.22	31.3	Breast
C31	50	F	11.65	29.2	Ovarian
C32	25	F	3.00	6.8	Pancreatic
C33	41	F	5.54	6.9	Cervical
C34	59	F	7.06	29.5	Breast
C35	49	F	30.08	13.9	Cervical

*. Nasopharyngeal cancer

a. Colon cancer

b. Rectal cancer

APPENDIX 2

Table 3. Descriptive Analysis on Serum 25(OH)D Levels in All Population of The Study

Descriptives				Statistic	Std. Error
Status					
25(OH)D (ng/mL)	Cancer	Mean		17.9340	1.82680
		95% Confidence Interval for Mean	Lower Bound	14.2215	
			Upper Bound	21.6465	
		5% Trimmed Mean		17.6029	
		Median		16.6200	
		Variance		116.802	
		Std. Deviation		10.80750	
		Minimum		3.00	
		Maximum		40.89	
		Range		37.89	
		Interquartile Range		21.08	
		Skewness		.333	.398
		Kurtosis		-.978	.778
	Healthy	Mean		15.1618	1.29723
		95% Confidence Interval for Mean	Lower Bound	12.5357	
			Upper Bound	17.7879	
		5% Trimmed Mean		14.5998	
		Median		12.5100	
		Variance		65.629	
		Std. Deviation		8.10119	
		Minimum		4.19	
		Maximum		42.69	
		Range		38.50	
		Interquartile Range		11.98	
		Skewness		1.218	.378
		Kurtosis		1.915	.741

Table 4. Kolmogorov-Smirnov Normality Test on Serum 25(OH)D Levels in All Population of The Study

Tests of Normality						
Status		Kolmogorov-Smirnov ^a			Shapiro-Wilk	
		Statistic	df	Sig.	Statistic	Sig.
25(OH)D (ng/mL)	Cancer	.121	35	.200*	.946	.084
	Healthy	.155	39	.019	.900	.002

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 5. Kolmogorov-Smirnov Normality Test on Serum 25(OH)D Levels in All Population of The Study After Lg10 Transformation

Lg10 Tests of Normality

Status	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
log_vitD Cancer	.120	35	.200 [*]	.923	35	.018
log_vitD Healthy	.082	39	.200 [*]	.984	39	.851

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 6. Independent Samples T-Test Result on Lg10 Transformed Serum 25(OD) Levels in All Population of The Study

Independent Samples Test									
		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
log_vitD	Equal variances assumed	5.534	.021	.436	72	.664	.02844	.06525	Lower: -.10164 Upper: .15852
	Equal variances not assumed			.427	59.296	.671	.02844	.06656	Lower: -.10473 Upper: .16160

APPENDIX 3

Table 7. Descriptive Analysis on Serum 25(OH)D Levels and Lymphocyte Count in Cancer Patients Group

Descriptives				
	Status		Statistic	Std. Error
25(OH)D (ng/mL)	Cancer	Mean	17.9340	1.82680
		95% Confidence Interval for Mean	Lower Bound Upper Bound	
			14.2215 21.6465	
		5% Trimmed Mean	17.6029	
		Median	16.6200	
		Variance	116.802	
		Std. Deviation	10.80750	
		Minimum	3.00	
		Maximum	40.89	
		Range	37.89	
		Interquartile Range	21.08	
		Skewness	.333	.398
		Kurtosis	-.978	.778
Lymphocyte Count (%)	Cancer	Mean	23.0829	2.49097
		95% Confidence Interval for Mean	Lower Bound Upper Bound	
			18.0206 28.1451	
		5% Trimmed Mean	21.7683	
		Median	22.5000	
		Variance	217.173	
		Std. Deviation	14.73678	
		Minimum	5.50	
		Maximum	66.00	
		Range	60.50	
		Interquartile Range	20.80	
		Skewness	1.113	.398
		Kurtosis	1.543	.778

Table 8. Shapiro-Wilk Normality Test on Serum 25(OH)D Levels and Lymphocyte Count in Cancer Patients Group

Tests of Normality							
		Kolmogorov-Smirnov ^a			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
25(OH)D (ng/mL)	Cancer	.121	35	.200 [*]	.946	35	.084
Lymphocyte Count (%)	Cancer	.116	35	.200 [*]	.900	35	.004

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 9. Shapiro-Wilk Normality Test on Lymphocyte Count in Cancer Patients Group After Lg10 Transformation

Tests of Normality						
Status	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
log_lymphocyte Cancer	.120	35	.200*	.949	35	.106

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure 10. Scatter/Dot Analysis for 25(OH)D and Lymphocyte Count in Cancer Patients Group

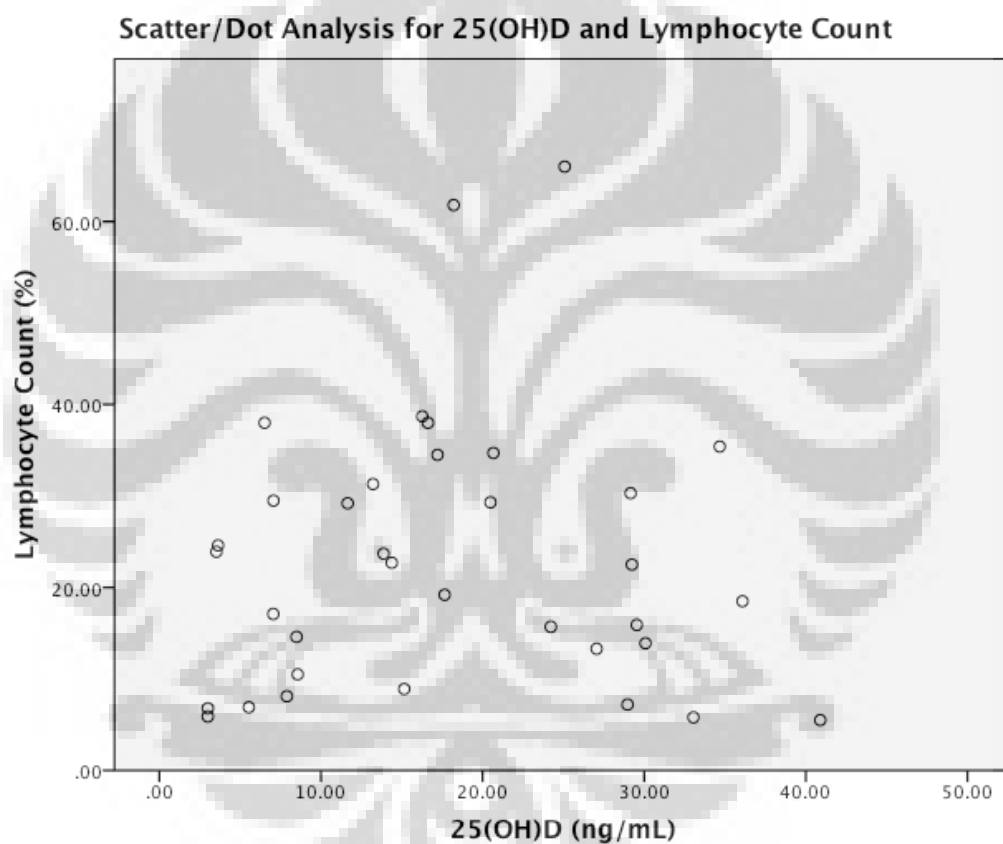


Table 10. Descriptive Analysis on Lymphocyte Count and Categorized Vitamin D Status in Cancer Patients Group

Descriptives				Statistic	Std. Error
Lymphocyte Count (%)		Vitamin D Category			
Lymphocyte Count (%)	Deficiency <10.00	Mean		21.0400	3.25173
		95% Confidence Interval for Mean	Lower Bound	14.2340	
			Upper Bound	27.8460	
		5% Trimmed Mean		19.3833	
		Median		16.5000	
		Variance		211.475	
		Std. Deviation		14.54219	
		Minimum		5.90	
		Maximum		66.00	
		Range		60.10	
		Interquartile Range		20.75	
		Skewness		1.599	.512
		Kurtosis		3.631	.992
	Insufficiency 10.01–29.99	Mean		28.2846	4.03283
		95% Confidence Interval for Mean	Lower Bound	19.4978	
			Upper Bound	37.0714	
		5% Trimmed Mean		27.6718	
		Median		29.2000	
		Variance		211.428	
		Std. Deviation		14.54057	
		Minimum		5.80	
		Maximum		61.80	
		Range		56.00	
		Interquartile Range		17.85	
		Skewness		.632	.616
		Kurtosis		1.319	1.191
	Sufficiency > 29.99	Mean		9.7000	4.20000
		95% Confidence Interval for Mean	Lower Bound	-43.6661	
			Upper Bound	63.0661	
		5% Trimmed Mean		.	
		Median		9.7000	
		Variance		35.280	
		Std. Deviation		5.93970	
		Minimum		5.50	
		Maximum		13.90	
		Range		8.40	
		Interquartile Range		.	
		Skewness		.	.
		Kurtosis		.	.

Table 11. Shapiro-Wilk Normality Test on Lymphocyte Count and Categorized Vitamin D Status in Cancer Patients Group

Vitamin D Category		Kolmogorov-Smirnov ^a			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Lymphocyte Count (%)	Deficiency <10.00	.157	20	.200	.856	20	.007
	Insufficiency 10.01–29.99	.160	13	.200*	.950	13	.597
	Sufficiency > 29.99	.260	2	.			

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 12. Shapiro-Wilk Normality Test on Lymphocyte Count and Categorized Vitamin D Status in Cancer Patients Group After Lg10 Transformation

Tests of Normality						
Vitamin D Category		Kolmogorov-Smirnov ^a			Shapiro-Wilk	
		Statistic	df	Sig.	Statistic	Sig.
log_lymphocyte	Deficiency <10.00	.115	20	.200*	.958	.498
	Insufficiency 10.01–29.99	.181	13	.200*	.906	.163
	Sufficiency >29.99	.260	2	.		

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 13. Homogeneity of Variances Test on Lymphocyte Count and Categorized Vitamin D Status in Cancer Patients Group and Categorized Vitamin D Status in Cancer Patients Group

Test of Homogeneity of Variances			
log_lymphocyte			
Levene Statistic	df1	df2	Sig.
.228	2	32	.798

Table 14. One-way ANOVA Test Result on Lg10 Transformed Lymphocyte Count and Categorized Vitamin D Status in Cancer Patients Group

ANOVA					
log_lymphocyte					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.416	2	.208	2.536	.095
Within Groups	2.626	32	.082		
Total	3.042	34			

APPENDIX 4

Table 15. Descriptive Analysis on 25(OH)D Levels and Gender Groups in

Descriptives				Statistic	Std. Error
Gender					
25(OH)D (ng/mL)	F	Mean		15.7837	1.87221
		95% Confidence Interval for Mean	Lower Bound	11.9353	
			Upper Bound	19.6321	
		5% Trimmed Mean		15.5108	
		Median		15.1500	
		Variance		94.640	
		Std. Deviation		9.72831	
		Minimum		3.00	
		Maximum		34.67	
		Range		31.67	
		Interquartile Range		18.02	
		Skewness		.351	.448
		Kurtosis		-1.084	.872
	M	Mean		25.1913	4.14997
		95% Confidence Interval for Mean	Lower Bound	15.3781	
			Upper Bound	35.0044	
		5% Trimmed Mean		25.3269	
		Median		26.6900	
		Variance		137.778	
		Std. Deviation		11.73789	
		Minimum		7.05	
		Maximum		40.89	
		Range		33.84	
		Interquartile Range		20.62	
		Skewness		-.262	.752
		Kurtosis		-1.149	1.481

Cancer Patients

Table 16. Shapiro-Wilk Normality Test on 25(OH)D Levels and Gender Groups in Cancer Patients

Tests of Normality						
Gender		Kolmogorov-Smirnov ^a			Shapiro-Wilk	
		Statistic	df	Sig.	Statistic	Sig.
25(OH)D (ng/mL)	F	.141	27	.176	.932	.075
	M	.132	8	.200*	.968	.881

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 17. Independent Samples T-Test Result on 25(OH)D Levels and Gender Groups in Cancer Patients

Independent Samples Test									
		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
25(OH)D (ng/mL)	Equal variances assumed	.503	.483	-2.294	33	.028	-9.40755	4.10096	-17.75102 -1.06407
	Equal variances not assumed			-2.066	10.028	.066	-9.40755	4.55274	-19.54791 .73282



APPENDIX 5

Table 18. Descriptive Analysis on 25(OH)D Levels and Different Age Groups in Cancer Patients

Descriptives				Statistic	Std. Error
Age Category					
25(OH)D (ng/mL)	20-30	Mean		14.0400	11.04000
		95% Confidence Interval for Mean	Lower Bound	-126.2365	
			Upper Bound	154.3165	
		5% Trimmed Mean		.	
		Median		14.0400	
		Variance		243.763	
		Std. Deviation		15.61292	
		Minimum		3.00	
		Maximum		25.08	
		Range		22.08	
		Interquartile Range		.	
		Skewness		.	.
		Kurtosis		.	.
	31-40	Mean		28.4583	5.26654
		95% Confidence Interval for Mean	Lower Bound	14.9203	
			Upper Bound	41.9964	
		5% Trimmed Mean		29.1470	
		Median		31.2950	
		Variance		166.418	
		Std. Deviation		12.90033	
		Minimum		3.63	
		Maximum		40.89	
		Range		37.26	
		Interquartile Range		13.59	
		Skewness		-1.823	.845
		Kurtosis		4.007	1.741
	41-50	Mean		15.4238	3.08801
		95% Confidence Interval for Mean	Lower Bound	8.1218	
			Upper Bound	22.7257	
		5% Trimmed Mean		15.2703	
		Median		15.7150	
		Variance		76.287	
		Std. Deviation		8.73422	
		Minimum		3.53	
		Maximum		30.08	
		Range		26.55	
		Interquartile Range		13.56	
		Skewness		.209	.752
		Kurtosis		-.340	1.481
	51-59	Mean		16.0774	2.12931
		95% Confidence Interval for Mean	Lower Bound	11.6039	
			Upper Bound	20.5509	
		5% Trimmed Mean		15.6926	
		Median		15.1500	
		Variance		86.145	
		Std. Deviation		9.28143	
		Minimum		3.00	
		Maximum		36.08	
		Range		33.08	
		Interquartile Range		16.33	
		Skewness		.651	.524
		Kurtosis		-.433	1.014

Table 19. Shapiro-Wilk Normality Test on 25(OH)D Levels and Different Age Groups in Cancer Patients

Tests of Normality						
Age Category	Kolmogorov-Smirnov ^a			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
25(OH)D (ng/mL) 20-30	.260	2	.			
31-40	.349	6	.021	.804	6	.064
41-50	.149	8	.200*	.963	8	.840
51-59	.170	19	.153	.926	19	.149

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 20. Homogeneity of Variances Test on 25(OH)D Levels and Different Age Groups in Cancer Patients

Test of Homogeneity of Variances			
25(OH)D (ng/mL)			
Levene Statistic	df1	df2	Sig.
.297	3	31	.827

Table 21. One-way ANOVA Test Result on 25(OH)D Levels and Different Age Groups in Cancer Patients

ANOVA					
25(OH)D (ng/mL)					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	810.801	3	270.267	2.651	.066
Within Groups	3160.470	31	101.951		
Total	3971.271	34			