EVALUATION OF SERUM LEVELS OF SOME ESSENTIAL TRACE ELEMENTS (CALCIUM, ZINC, COPPER, SELENIUM, AND VITAMIN D AND K) IN PATIENTS DIAGNOSED WITH OSTEOARTHRITIS

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JANUARY, 2020.

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BEING A THESIS IN THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS DEGREE IN MEDICAL LABORATORY SCIENCE (CLINICAL CHEMISTRY) UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA.

JANUARY, 2020.

CERTIFICATION

This is to certify that this project work was carried out by **AJILEYE SAMUEL ADEOLA** under our supervision, in Partial fulfillment for the requirement for Award of Masters of Science in Clinical Chemistry, Department of Medical Laboratory Science.

_____ _____

PROF. M.A. EMOKPAE **DATE**
Supervisor

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DR. F.O. AKINBO **DATE**
Head of Department

_____ _____

EXTERNAL EXAMINER **DATE**

DEDICATION

To God Almighty, the source of all Knowledge, wisdom, and understanding, who graciously inspired me throughout the course of this work.

ACKNOWLEDGMENT

I sincerely wish to acknowledge the invaluable and painstaking effort shown by my supervisor, Prof. M.A. Emokpae for his meticulous supervision, contributions, and dedication to the success of this work.

My appreciation also goes to my amiable Head of Department Dr. F.O. Akinbo, my big Daddy, Prof I.N. Ibeh, Prof. H.B. Osadolor, and Dr. G.I. Eze and all the lecturers in the Department of Medical Laboratory Science, Benin City.

I am greatly indebted to my Lovely Wife Her excellences Mrs Anike Mercy Ajileye, for the encouragement and moral support I received from her throughout the course of my study and this research work.

Moreover, my sincere appreciation also goes to Members of my family Mr Ajileye Ayodeji for his brotherly support, my mother Mrs M.T. Ajileye, Mrs B.F Aiyelari, Mrs Adeleye A and a host of others for their effort and support to ensure the completion of this work.

I also appreciate my course mates most especially Mr Fidelis Oyakhire, Mr. Mathias Adeleye, Ogbonaya E, Ogunmola Emmanuel and A host of my colleagues at work most especially all staff of medical laboratory science Department General hospital Ipe Akoko for their love, supports and many helpful comments during the project work.

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ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis and is a leading cause of pain and disability worldwide. Several biological structural trace elements involved in various vital metabolic processes relating to health and diseases include calcium and zinc has been proved to have a role in decreasing inflammatory pain, joint stiffness and other disabling symptoms associated with osteoarthritis. The purpose of the present study was to estimate the serum levels of some essential trace element such as calcium, copper, zinc, selenium and vitamin D and K in elderly patients with OA. A total of 300 patients comprising of one hundred and fifty OA subjects and 150 non osteoarthritis subjects were recruited for this study. Atomic absorption spectroscopy were used to measure the serum concentrations of Ca, Cu, Zn, Se, after it has been properly digested with its specific solution while Vitamin D and K was measured using HPLC and spectrophotometer respectively after treating with its specific solution and the results were compared with those of healthy controls. The socio-demographics characteristic of osteoarthritis and non-osteoarthritis subjects depicts no significant difference when compared with measured values across all age group. Majority of the subjects with osteoarthritis were female than male with a percentage difference of 60.7% and 39.3% respectively. The measured mineral concentration (calcium, copper and zinc) of osteoarthritis were significantly lower ($p<0.001$) than non-osteoarthritis subject between the distributions ($p<001$). However, zinc was not significant ($P = 0.121$). The results showed that 92/150(61.3%) of subjects with osteoarthritis had calcium level below the reference range, 113/150(75.3%) of copper, 30/150(20.0%) of zinc and 25/150(16.7%) of selenium were below normal reference range. Similarly, among non-osteoarthritis subjects tested the results showed that 40/150(26.7%) of the subjects had calcium level below the reference range, 69/150(46.0%) of copper, 20/150(13.3%) of zinc and 8/150(5.3%) of selenium were below normal reference range. The levels of vitamins D and K between osteoarthritis and non-osteoarthritis control groups showed that about 19.3% of the cases with osteoarthritis had a low level of Vitamin K compared to only 9.3% of the apparently healthy controls. This difference was found to be statistically significant ($P < 0.013$). Also, 30.7% and 5.3% of osteoarthritis and non-osteoarthritis subjects respectively had low level of vitamin D ($P < 0.001$). Osteoarthritis is not age dependent, the proportion of subjects within age group of 61-70 years had the highest percentage of osteoarthritis (56.0%). While the subjects within age group 70-80 years old had the lowest 5.3% of osteoarthritis. Means of all parameters compared were all found to be significantly different at 0.05 level except age which shows a very high significant difference ($P > 0.116$) between osteoarthritis and non-osteoarthritis subjects. It is therefore recommended that all patients with osteoarthritis should routine undergo structural trace element measurement.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Osteoarthritis (OA) is a type of joint disease that results from the breakdown of joint cartilage and underlying bone (Arden *et al*., 2015). The most common symptoms are joint pains and stiffness (Wang *et al*., 2012). Initially, symptoms may occur only following exercise, but over time may become constant (Wang *et al*., 2012). Other symptoms may include joint swelling, decreased range of motion, weakness or numbness of the arms and legs (Wang *et al*., 2012). The total economic burden for arthritis is estimated to be 1%–2.5% of the gross national product in Western countries. Osteoarthritis is a leading cause of disability, affecting 60 - 70% of people aged ≥ 60 years. Multiple etiologies are suspected to contribute to the formation of OA, including defective articular cartilage structure, biosynthesis, joint trauma, joint instability, inflammatory conditions, congenital and developmental abnormalities (Wang *et al*., 2012). It is sometimes called degenerative joint disease or degenerative arthritis, osteoarthritis is the most common chronic condition of the joints. The prevalence of osteoarthritis in Nigeria is estimated to be 0.4% of the population among adults aged 65years across Africa (Silman *et al*., 1993), OA is found as the most prevalence arthritis in urban settings this was found to be 55.1% and in rural settings all was found in South Africa ranged from 29.5%, 29.7%, up to 82.7% among adults aged 65years (Bija *et al*., 2014). Other urban Hospital-based studies reporting OA of the knee are Burkina Faso with a prevalence of 0.5% among adults, Tunisia reported prevalence of 4.7% of knee osteoarthritis among elderly subjects and 9.9% prevalence with musculoskeletal condition in Cameroon (Ouedraogo *et al*., 2010). Approximately 27 million Americans are affected with osteoarthritis. It is the most common form of arthritis, affecting about 237 million (3.3% of the

population) (March *et al*., 2014; GBD, 2015). About 10% of males and 18% of females are affected (Glyn-Jones *et al*., 2015). It is the cause of about 2% of years lived with disability (March *et al*., 2014). In Australia, about 1.9 million people are affected (Elsternwick, 2013), and in the United States, 30 to 53 million people are affected (CDCP, 2016; Cisternas *et al*., 2016). It becomes more common in both sexes as people become older (Wang *et al*., 2012). OA can affect any joint, but it occurs most often in knees, hips, lower back and neck, small joints of the fingers and the bases of the thumb and big toe. The pain is naturally made worse by prolonged activity and relieved by rest. Stiffness is most common in the morning, and typically lasts less than thirty minutes after beginning daily activities, but may return after periods of inactivity. Osteoarthritis can cause a crackling noise (called "crepitus") when the affected joint is moved, especially shoulder and knee joint. A person may also complain of joint locking and joint instability. These symptoms would affect their daily activities due to pain and stiffness (Sinusas, 2012). Osteoarthritis commonly affects the hands, feet, spine, and the large weight-bearing joints, such as the hips and knees, although in theory, any joint in the body can be affected. As osteoarthritis progresses, movement patterns (such as gait), are typically affected (Vincent *et al*., 2012). Osteoarthritis is the most common cause of a joint effusion of the knee (Mayo Clinic, 2017). In smaller joints, such as the fingers, hard bony enlargements, called Heberden's nodes (on the distal interphalangeal joints) or Bouchard's nodes (on the proximal interphalangeal joints), may form, and though they are not necessarily painful, they do limit the movement of the fingers significantly. Osteoarthritis of the toes may be a factor causing formation of bunions (Mayo Clinic 2016), rendering them red or swollen. In normal joints, a firm, rubbery material called cartilage covers the end of each bone. Cartilage provides a smooth, gliding surface for joint motion and acts as a cushion between the bones. The most commonly

involved joints are those near the ends of the fingers, at the base of the thumb, neck, lower back, knee, and hips (Wang *et al*., 2012). Risk factor is greater in those who are overweight, have one leg of a different length, and have jobs that result in high levels of joint stress (Wang *et al*., 2012; Glyn-Jones *et al*., 2015; Vingård *et al*., 2016). Osteoarthritis is believed to be caused by mechanical stress on the joint and low grade inflammatory processes (Berenbaum, 2013). It develops as cartilage is lost and the underlying bone becomes affected (Wang *et al*., 2012), as pain may make it difficult to exercise, muscle loss may occur (Conaghan, 2014; Glyn-Jones *et al*., 2015). Diagnosis is typically based on signs and symptoms, with medical imaging and other tests occasionally used to either support or rule out other problems (Wang *et al*., 2012) In contrast to rheumatoid arthritis, which is primarily an inflammatory condition, in osteoarthritis, the joints do not become hot or red (Wang *et al*., 2012).

Damage from mechanical stress with insufficient self repair by joints is believed to be the primary cause of osteoarthritis (Brandt *et al*., 2009). Sources of this stress may include misalignments of bones caused by congenital or pathogenic causes; mechanical injury; excess body weight; loss of strength in the muscles supporting a joint; and impairment of peripheral nerves, leading to sudden or uncoordinated movements (Brandt *et al*., 2009). However, exercise including running in the absence of injury has not been found to increase the risk of knee osteoarthritis (Bosomworth, 2009). Nor has cracking one's knuckles been found to play a role (Deweber *et al*., 2011).

A number of studies have shown that there is a greater prevalence of the disease among siblings and especially identical twins, indicating a hereditary basis (Valdes *et al.,* 2008). Although a single factor is not generally sufficient to cause the disease, about half of the variations in susceptibility has been assigned to genetic factors (Spector and MacGregor, 2004).

As early human ancestors evolved into two legged animal, changes occurred in the pelvis, hip joint and spine which increased the risk of osteoarthritis (Hogervorst *et al*., 2009). Additionally, genetic variations that increase the risk were likely not selected again because usually problems only occur after reproductive success (Van der Kraan and van den Berg, 2008). The development of osteoarthritis is correlated with a history of previous joint injury and with obesity, especially with respect to knees (Coggon *et al*., 2001). Since the correlation with obesity has been observed not only for knees but also for non-weight bearing joints and the loss of body fat is more closely related to symptom relief than the loss of body weight, it has been suggested that there may be a metabolic link to body fat as opposed to just mechanical loading (Pottie *et al*., 2006).

Changes in sex hormone levels may play a role in the development of osteoarthritis as it is more prevalent among post-menopausal women than among men of the same age (Tanamas *et al*., 2011; Linn *et al*., 2012). A study of mice found natural female hormones to be protective while i njections of the male hormone dihydrotestosterone reduced protection (Ma *et al*., 2007).

Increased risk of developing knee and hip osteoarthritis was found among those who work with manual handling (e.g. lifting), have physically demanding work, walk at work, and have climbing tasks at work (e.g. climb stairs or ladders) (Vingård *et al*., 2016). With hip osteoarthritis in particular, increased risk of development over time was found among those who work in bent or twisted positions (Vingård *et al*., 2016). For knee osteoarthritis in particular, increased risk was found among those who work in a kneeling or squatting position, experience heavy lifting in combination with a kneeling or squatting posture, and work standing up (Vingård *et al*., 2016). Women and men have similar occupational risks for the development of osteoarthritis (Vingård *et al*., 2016).

While osteoarthritis is a degenerative joint disease that may cause gross cartilage loss and morphological damage to other joint tissues. More subtle biochemical changes occur in the earliest stages of osteoarthritis progression. The water content of healthy cartilage is finely balanced by compressive force driving water out and hydrostatic and osmotic pressure drawing water in (Maroudas, 1976; Sanchez-Adams *et al.,* 2014). Collagen fibres exert the compressive force, whereas the Gibbs–Donnan effect and cartilage proteoglycans create osmotic pressure which tends to draw water in (Maroudas, 1976). However, during onset of osteoarthritis, the collagen matrix becomes more disorganized and there is a decrease in proteoglycan content within cartilage. The breakdown of collagen fibers results in a net increase in water content (Bollet and Nance, 1966; Mankin and Thrasher, 1975; Brocklehurst *et al*., 1984; Grushko *et al*., 1989; Chou *et al*., 2009). This increase occurs because whilst there is an overall loss of proteoglycans (and thus a decreased osmotic pull) (Venn and Maroudas, 1977; Brocklehurst *et al*., 1984), it is outweighed by a loss of collagen (Maroudas, 1976; Venn and Maroudas, 1977). Without the protective effects of the proteoglycans, the collagen fibers of the cartilage can become susceptible to degradation and thus exacerbate the degeneration. Inflammation of the synovium (joint cavity lining) and the surrounding joint capsule can also occur, though often mild (compared to the synovial inflammation that occurs in rheumatoid arthritis).

Other structures within the joint can also be affected (Madry *et al*., 2012). The ligaments within the joint become thickened and fibrotic and the menisci can become damaged and wear away (Englund *et al*., 2012). Menisci can be completely absent by the time a person undergoes a joint replacement. New bone outgrowths, called "spurs" or osteophytes, can form on the margins of the joints, possibly in an attempt to improve the congruence of the articular

cartilage surfaces in the absence of the menisci. The subchondral bone volume increases and becomes less mineralized (hypomineralization) (Li *et al*., 2013). All these changes can cause problems functioning. The pain in an osteoarthritic joint has been related to thickened synovium (Hill *et al*., 2001) and subchondral bone lesions (Felson *et al*., 2001).

Globally, as of 2010, approximately 250 million people had osteoarthritis of the knee (3.6% of the population) (Cross *et al*., 2014; Vos *et al*., 2012). Hip osteoarthritis affects about 0.85% of the population (Cross *et al*., 2014). As of 2004, osteoarthritis globally causes moderate to severe disability in 43.4 million people (GBD, 2004). Together, knee and hip osteoarthritis had a ranking for disability globally of 11th among 291 disease conditions assessed (Cross *et al*., 2014). As of 2012, osteoarthritis affected 52.5 million people in the United States, approximately 50% of whom were 65 years or older (*C*DCP, 2016). It is estimated that 80% of the population have radiographic evidence of osteoarthritis by age 65, although only 60% of those will have symptoms (Green, 2001). The rate of osteoarthritis in the United States is forecast to be 78 million (26%) adults by 2040 (*C*DCP, 2016).

There are ongoing efforts to determine if there are agents that modify outcomes in osteoarthritis. Sprifermin is one candidate drug. There is also tentative evidence that strontium ranelate may decrease degeneration in osteoarthritis and improve outcomes (Civjan, 2012; Bruyère *et al*., 2008).

As well as attempting to find disease-modifying agents for osteoarthritis, there is emerging evidence that a system-based approach is necessary to find the causes of osteoarthritis (Chu and Andriacchi, 2015). Changes may occur before clinical disease is evident due to abnormalities in biomechanics, biology or structure of joints that predispose them to develop clinical disease. Research is thus focusing on defining these early pre-osteoarthritis changes using biological,

mechanical, and imaging markers of osteoarthritis risk, emphasising multi-disciplinary approaches, and looking into personalized interventions that can reverse osteoarthritis risk in healthy joints before the disease becomes evident.

Guidelines outlining requirements for inclusion of soluble biomarkers in osteoarthritis clinical trials were published in 2015, (Kraus *et al*., 2015) but as of 2015, there are no validated biomarkers for osteoarthritis. One problem with using a specific type II collagen biomarker from the breakdown of articular cartilage is that the amount of cartilage is reduced (worn away) over time with progression of the disease. As a result, a patient can eventually have very advanced osteoarthritis with none of this biomarker detectable in their urine. Another problem with a systemic biomarker is that a patient can have osteoarthritis in multiple joints at different stages of disease at the same time, so the biomarker source cannot be determined. Some other collagen breakdown products in the synovial fluid correlated with each other after acute injuries (a known cause of secondary osteoarthritis) but did not correlate with the severity of the injury (Kumahashi *et al*., 2015).

OA occurs in people of all ages, osteoarthritis is most common in people older than 60 years of age. Common risk factors include increasing age, obesity, previous joint injury, overuse of the joint, weak thigh muscles, and genes. Metal concentrations in bones reflect long-term exposure, yet there is no evidence that would determine whether the mobilization of bone stores could occur so quickly that it may result in poisoning. Due to the characteristics and long recovery time of bone tissue, it may reflect a chronic level of exposure and serve as a basis of indirect assessment of environmental exposure. Among the elements necessary for life, the concentrations of zinc (Zn) and copper (Cu) are often determined in highly mineralized tissues. It was found that zinc accelerates bone formation and is essential for the correct ossification and

mineralization of the skeleton, especially the femoral epiphysis. Zinc and Copper are involved in the formation and metabolism of bone tissue. Naturally occurring minerals such as Calcium (Ca), copper (Cu), selenium (Se) and zinc (Zn) have shown anti-inflammatory effects in both animal and human studies. Animal model of OA, a deficiency of dietary Mg was shown to accelerate cartilage damage (Shakibaei *et al*., 1996). Copper is an essential cofactor in enzymes such as super oxide dismutase (SOD) that also needs Zn and Mn as cofactors. Many studies revealed a role for oxidative stress in the pathogenesis of OA, whereby ROS generation and impaired antioxidant status of the joint might result in the degradation of cartilage joint remodeling (Henrotin *et al*., 2005). Selenium is also an essential co-factor for glutathione peroxidase which may have a role in reducing the incidence of osteoarthritic lesion (Kurz *et al*., 2002). It is not known whether trace element status leads to disease or whether diseases set in due to the deficiency of trace elements. Although it is generally believed that a strict metabolic control delays the development of late complications OA.

1.2 Statement of Problem

Many trace elements have been recognized to play an important role in the pathogenesis and progression of many diseases, including osteoarthritis. However, prevalence data on arthritis in Africa is very scarce despite the overwhelming report on the rising prevalence of musculoskeletal, data from Africa are lacking and underestimated.in estimating the burden for osteoarthritis in Africa, only one study from south Africa was used emphasysing the pausating data in Africa (Symmond *et al*., 2006). More studies are needed to address the prevalence and true burden of this disease in Africa. Hence, investigating changes in the metabolism of these elements were the major reason for this reasearch.

1.3 Justification of Study

There has been increased interest recently in the incidence of Osteoarthritis in elderly subject resulting in progressive degenerative changes in the cartilage and articular tissues. Multiple etiologies are suspected to contribute to the formation of OA, including defective articular cartilage structure and biosynthesis, joint trauma, joint instability, congenital and developmental abnormalities, and inflammatory conditions. Measuring the levels of Ca, Cu, Zn, Se and some essential vitamin necessary for Oxidative damage is essential in understtanding cell dysfuction and degradation caused by oxygen free radicals in the pathobiology of degenerative joint disease

1.4 Aim of the Study

The aim of the study was to evaluate the concentrations of some essential trace elements deficiencies in patients diagnosed with Osteoarthritis

1.5 Specific Objectives

1. Assess and compare the age and gender distributions of the study population
2. Quantitatively determine the amount of Calcium (Ca), Zinc (Zn), Copper (Cu), Selenium (Se) in patients diagnosed with Osteoarthritis
3. Evaluate the amount of vitamin D and K in the patients serum sample
4. Assess and compare the data obtained from analysis with control serum of Non Osteoarthritis patients
5. To provide information to the researcher in developing therapeutic approach to solving problem associated with osteoarthritis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Calcium

Calcium (Ca) is a chemical element with atomic number 20. It is an alkaline earth metal, calcium is a reactive pale yellow metal that forms a dark oxide-nitride layer when exposed into the environment or in air (Meija *et al.,* 2016). Its physical and chemical properties are most similar to its heavier homologues strontium and barium. It is the fifth most abundant element in Earth's crust and the third most abundant metal, after iron and aluminium. The second common calcium compound on Earth is calcium carbonate, found in limestone and the fossilised remnants of early sea life; gypsum, anhydrite, fluorite, and apatite are also sources of calcium (Greenwood and Earnshaw 2005).

The name is derives from Latin *calx* "lime", which was obtained from heating limestone. Its compounds were known to the ancients, though their chemistry was unknown until the seventeenth century. It was isolated by Humphry Davy in 1808 via electrolysis of its oxide, who named the element. While the pure metal does not have many applications due to its high reactivity, it is often used as an alloying component in small quantities in steelmaking, and calcium–lead alloys are sometimes used in automotive batteries. Calcium compounds on the other hand are very widely used in many industries: for example, they are used in foods and pharmaceuticals for calcium supplementation, in the paper industry as bleaches, in cement, in the manufacture of soaps, and as electrical insulators.

Calcium ions play a vital role in the physiology and biochemistry of organisms and the cell as electrolytes. They also play a role in signal transduction pathways, where they act as a second messenger, in neurotransmitter release from neurons, in contraction of all muscle cell types, and in fertilization. Several enzymes need calcium ions as a cofactor. Calcium ions outside cells are

also important for maintaining the potential difference across excitable cell membranes, as well as proper bone formation.

2.1.1 Characteristics of Calcium

Calcium is a very ductile silvery metal with a pale yellow tint whose properties are very similar to the heavier elements in its group, strontium, barium, and radium. A calcium atom has twenty electrons, arranged in the electron configuration $[Ar]4s^2$. Like the other elements placed in group 2 of the periodic table, calcium has two valence electrons in the outermost s-orbital, which are very easily lost in chemical reactions to form a dipositive ion with the stable electron configuration of a noble gas, in this case argon. Hence, calcium is almost always divalent in its compounds, which are usually ionic. Hypothetical univalent salts of calcium would be stable with respect to their elements, but not to disproportionate to the divalent salts and calcium metal, because the enthalpy of formation of MX_2 is much higher than those of the hypothetical MX. This occurs because of the much greater lattice energy afforded by the more highly charged Ca^{2+} cation compared to the hypothetical Ca^{+} cation (Greenwood and Earnshaw, 2005).

Calcium is considered to be an alkaline earth metal, along with these heavier elements and the lighter beryllium and magnesium. Nevertheless, there are significant differences in chemical and physical properties between beryllium and magnesium (which behave more like aluminium and zinc respectively and have some of the weaker metallic character of the post-transition metals) and the group members from calcium onwards, which traditionally led to "alkaline earth metal" only applying to the latter group (Parish*,* 1977). This classification is mostly obsolete in English-language sources, but is still used in other countries such as Japan (Fukuma*,* 2013*).* As a result,

comparisons with strontium and barium are more germane to calcium chemistry than comparisons with magnesium (Greenwood and Earnshaw 2005).

2.1.2 Physical Properties of Calcium

Calcium metal melts at 842 °C and boils at 1494 °C, higher than its adjacent group 2 metals do. It crystallises in the face-centered cubic arrangement like strontium; above 450 °C, it changes to an anisotropic hexagonal close-packed arrangement like magnesium. The density of 1.55 $g \cdot cm^{-3}$ is the lowest in its group, with others decreasing towards it (Greenwood and Earnshaw, 2005). Calcium can be cut with a knife with effort, although it is still harder than lead. While calcium is a poorer conductor of electricity than copper or aluminium by volume, it is a better conductor than both of them by mass due to its very low density (Ropp, 2012). Although it is infeasible for terrestrial applications as it reacts quickly with atmospheric oxygen, its use as a conductor in space has been considered (Hluchan and Pomerantz 2005).

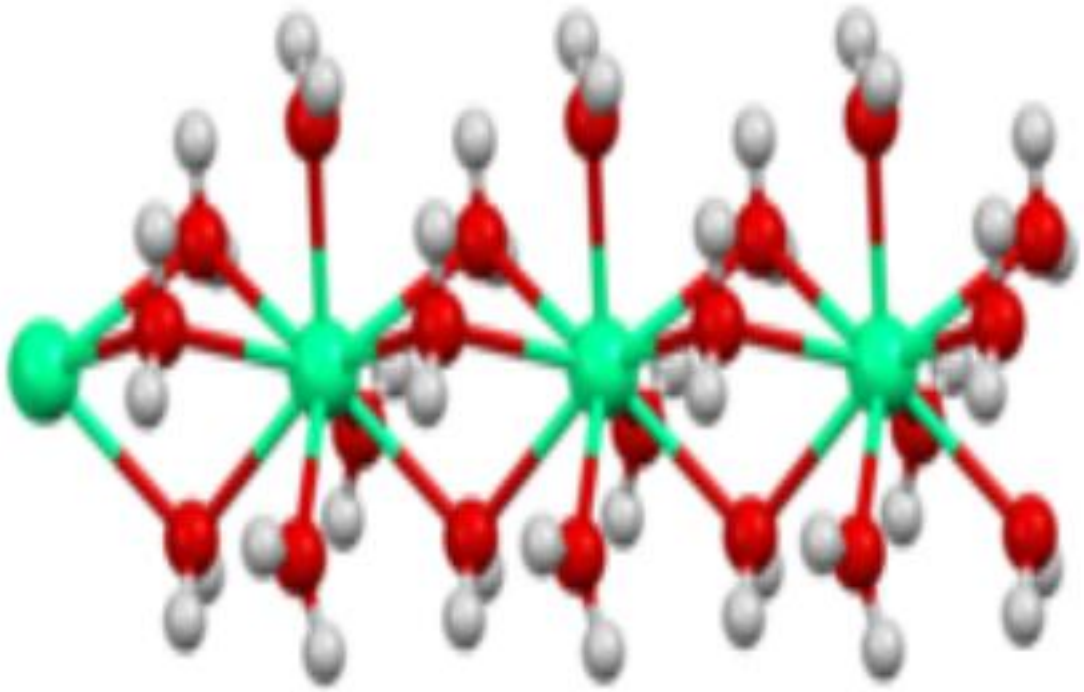


Figure 2.1: Structure of the polymeric $[Ca(H_2O)_6]^{2+}$ center in hydrated calcium chloride, illustrating the high coordination number typical for calcium complexes (Greenwood and Earnshaw 2005).

The chemistry of calcium is that of a typical heavy alkaline earth metal. For example, calcium spontaneously reacts with water more quickly than magnesium and less quickly than strontium to produce calcium hydroxide and hydrogen gas. It also reacts with the oxygen and nitrogen in the air to form a mixture of calcium oxide and calcium nitride (Hammond, 2005). When finely divided, it spontaneously burns in air to produce the nitride. In bulk, calcium is less reactive: it quickly forms a hydration coating in moist air, but below 30% relative humidity it may be stored indefinitely at room temperature (Hluchan and Pomerantz, 2005).

Besides the simple oxide CaO, the peroxide CaO_2 can be made by direct oxidation of calcium metal under a high pressure of oxygen, and there is some evidence for a yellow superoxide $Ca(O_2)_2$ (Greenwood and Earnshaw, 2005). Calcium hydroxide, $Ca(OH)_2$, is a strong base, though it is not as strong as the hydroxides of strontium, barium or the alkali metals (Greenwood and Earnshaw 2005). All four dihalides of calcium are known. (Greenwood and Earnshaw, 2005). Calcium carbonate ($CaCO_3$) and calcium sulfate ($CaSO_4$) are particularly abundant minerals (Greenwood and Earnshaw, 2005). Like strontium and barium, as well as the alkali metals and the divalent lanthanides europium and ytterbium, calcium metal dissolves directly in liquid ammonia to give a dark blue solution (Greenwood and Earnshaw, 2005).

Due to the large size of the Ca^{2+} ion, high coordination numbers are common, up to 24 in some intermetallic compounds such as $CaZn_{13}$ (Greenwood and Earnshaw, 2005). Calcium is readily complexed by oxygen chelates such as EDTA and polyphosphates, which are useful in analytic chemistry and removing calcium ions from hard water. In the absence of steric hindrance, smaller group 2 cations tend to form stronger complexes, but when large polydentate macrocycles are involved a reverse might result (Greenwood and Earnshaw, 2005).

Although calcium is in the same group as magnesium and organomagnesium compounds are very commonly used throughout chemistry, organocalcium compounds are not similarly widespread because they are more difficult to make and more reactive, although they have recently been investigated as possible catalysts (Harder *et al.,* 2001; Crimmin *et al.,* 2005; Jenter *et al.,* 2011; Arrowsmith *et al.*, 2011*;* Penafiel *et al*., 2014). Organocalcium compounds tend to be more similar to organoytterbium compounds due to the similar ionic radii of Yb^{2+} (102 pm) and Ca^{2+} (100 pm). Most of these compounds can only be prepared at low temperatures; bulky ligands tend to favor stability. For example, calcium dicyclopentadienyl, $Ca(C_5H_5)_2$, must be made by directly reacting calcium metal with mercurocene or cyclopentadiene itself; replacing the C_5H_5 ligand with the bulkier $C_5(CH_3)_5$ ligand on the other hand increases the compound's solubility, volatility, and kinetic stability (Greenwood and Earnshaw, 2005).

2.1.3 Isotopes of Calcium

Natural calcium is a mixture of five stable isotopes (^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, and ^{46}Ca) and one isotope with a half-life so long that it can be considered stable for all practical purposes (^{48}Ca, with a half-life of about 4.3×10^{19} years). Calcium is the first (lightest) element to have six naturally occurring isotopes (Hammond, 2005).

By far the most common isotope of calcium in nature is ^{40}Ca, which makes up 96.941% of all natural calcium. It is produced in the silicon-burning process from fusion of alpha particles and is the heaviest stable nuclide with equal proton and neutron numbers; its occurrence is also supplemented slowly by the decay of primordial ^{40}K. Adding another salpha particle would lead to unstable ^{44}Ti, which quickly decays via two successive electron captures to stable ^{44}Ca; this makes up 2.806% of all natural calcium and is the second-most common isotope. The other four

natural isotopes, ^{42}Ca, ^{43}Ca, ^{46}Ca, and ^{48}Ca, are significantly rarer, each comprising less than 1% of all natural calcium. The four lighter isotopes are mainly products of the oxygen-burning and silicon-burning processes, leaving the two heavier ones to be produced via neutron-capturing processes. ^{46}Ca is mostly produced in a "hot" s-process, as its formation requires a rather high neutron flux to allow short-lived ^{45}Ca to capture a neutron. ^{48}Ca is produced by electron capture in the r-process in type Ia supernovae, where high neutron excess and low enough entropy ensures its survival (Cameron*,* 1973; Clayton*,* 2003).

^{46}Ca and ^{48}Ca are the first "classically stable" nuclides with a six-neutron or eight-neutron excess respectively. Although extremely neutron-rich for such a light element, ^{48}Ca is very stable because it is a doubly magic nucleus, having 20 protons and 28 neutrons arranged in closed shells. Its beta decay to ^{48}Sc is very hindered because of the gross mismatch of nuclear spin: ^{48}Ca has zero nuclear spin, being even–even, while ^{48}Sc has spin 6+, so the decay is forbidden by the conservation of angular momentum. While two excited states of ^{48}Sc are available for decay as well, they are also forbidden due to their high spins. As a result, when ^{48}Ca does decay, it does so by double beta decay to ^{48}Ti instead, being the lightest nuclide known to undergo double beta decay (Audi *et al.,* 2003; Arnold *et al.,* 2016). The heavy isotope ^{46}Ca can also theoretically undergo double beta decay to ^{46}Ti as well, but this has never been observed; the lightest and most common isotope ^{40}Ca is also doubly magic and could undergo double electron capture to ^{40}Ar, but this has likewise never been observed. Calcium is the only element to have two primordial doubly magic isotopes. The experimental lower limits for the half-lives of ^{40}Ca and ^{46}Ca are 5.9×10^{21} years and 2.8×10^{15} years respectively (Audi *et al.,* 2003).

Apart from the practically stable ^{48}Ca, the longest lived radioisotope of calcium is ^{41}Ca. It decays by electron capture to stable ^{41}K with a half-life of about a hundred thousand years. Its existence

in the early Solar System as an extinct radionuclide has been inferred from excesses of ^{41}K: traces of ^{41}Ca also still exist today, as it is a cosmogenic nuclide, continuously reformed through neutron activation of natural ^{40}Ca (Clayton, 2003). Many other calcium radioisotopes are known, ranging from ^{34}Ca to ^{57}Ca: they are all much shorter-lived than ^{41}Ca, the most stable among them being ^{45}Ca (half-life 163 days) and ^{47}Ca (half-life 4.54 days). The isotopes lighter than ^{42}Ca usually undergo beta plus decay to isotopes of potassium, and those heavier than ^{44}Ca usually undergo beta minus decay to isotopes of scandium, although near the nuclear drip lines proton emission and neutron emission begin to be significant decay modes as well (Audi *et al.,* 2003).

Like other elements, a variety of processes alter the relative abundance of calcium isotopes (Russell *et al.,* 1978). The best studied of these processes is the mass-dependent fractionation of calcium isotopes that accompanies the precipitation of calcium minerals such as calcite, aragonite and apatite from solution. Lighter isotopes are preferentially incorporated into these minerals, leaving the surrounding solution enriched in heavier isotopes at a magnitude of roughly 0.025% per atomic mass unit (amu) at room temperature. Mass-dependent differences in calcium isotope composition are conventionally expressed by the ratio of two isotopes (usually $^{44}Ca/^{40}Ca$) in a sample compared to the same ratio in a standard reference material. $^{44}Ca/^{40}Ca$ varies by about 1% among common earth materials (Skulan and Depaolo 1999).

2.1.4 Occurrence and Production of Calcium

At 3%, calcium is the fifth most abundant element in the Earth's crust, and the third most abundant metal behind aluminium and iron (Greenwood and Earnshaw, 2005). It is also the fourth most abundant element in the lunar highlands (Greenwood and Earnshaw, 2005). Sedimentary calcium carbonate deposits pervade the Earth's surface as fossilised remains

of past marine life; they occur in two forms, the rhombohedral calcite (more common) and the orthorhombic aragonite (forming in more temperate seas). Minerals of the first type include limestone, dolomite, marble, chalk, and iceland spar; aragonite beds make up the Bahamas, the Florida Keys, and the Red Sea basins. Corals, sea shells, and pearls are mostly made up of calcium carbonate. Among the other important minerals of calcium are gypsum ($CaSO_4 \cdot 2H_2O$), anhydrite ($CaSO_4$), fluorite (CaF_2), and apatite ($[Ca_5(PO_4)_3F]$) (Greenwood and Earnshaw 2005). The major producers of calcium are China (about 10000 to 12000 tonnes per year), Russia (about 6000 to 8000 tonnes per year), and the United States (about 2000 to 4000 tonnes per year). Canada and France are also among the minor producers. In 2005, about 24000 tonnes of calcium were produced; about half of the world's extracted calcium is used by the United States, with about 80% of the output used each year (Hluchan and Pomerantz, 2005). In Russia and China, Davy's method of electrolysis is still used, but is instead applied to molten calcium chloride (Hluchan and Pomerantz 2005). Since calcium is less reactive than strontium or barium, the oxide–nitride coating that results in air is stable and lathe machining and other standard metallurgical techniques are suitable for calcium (Greenwood and Earnshaw, 2005). In the United States and Canada, calcium is instead produced by reducing lime with aluminium metal at high temperatures (Hluchan and Pomerantz, 2005).

2.1.5 Uses of Calcium

The largest use of calcium is in steelmaking, due to its strong chemical affinity for oxygen and sulfur. Its oxides and sulfides, once formed, give liquid lime aluminate and sulfide inclusions in steel which float out; on treatment, these inclusions disperse throughout the steel and became small and spherical, improving castability, cleanliness and general mechanical properties.

Calcium is also used in maintenance-free automotive batteries, in which the use of 0.1% calcium-lead alloys instead of the usual antimony–lead alloys leads to lower water loss and lower self-discharging. Due to the risk of expansion and cracking, aluminium is sometimes also incorporated into these alloys. These lead–calcium alloys are also used in casting, replacing lead–antimony alloys (Hluchan and Pomerantz, 2005). Calcium is also used to strengthen aluminium alloys used for bearings, for the control of graphitic carbon in cast iron, and to remove bismuth impurities from lead (Greenwood and Earnshaw, 2005). Calcium metal is found in some drain cleaners, where it functions to generate heat and calcium hydroxide that saponifies the fats and liquefies the proteins (for example, those in hair) that block drains (Rumack, 2010). Besides metallurgy, the reactivity of calcium is exploited to remove nitrogen from high-purity argon gas and as a getter for oxygen and nitrogen. It is also used as a reducing agent in the production of chromium, zirconium, thorium, and uranium. It can also be used to store hydrogen gas, as it reacts with hydrogen to form solid calcium hydride, from which the hydrogen can easily be re-extracted (Greenwood and Earnshaw, 2005).

Calcium isotope fractionation during mineral formation has led to several applications of calcium isotopes. In particular, the 1997 observation by Skulan and DePaolo (Skulan *et al.,* 1997). that calcium minerals are isotopically lighter than the solutions from which the minerals precipitate is the basis of analogous applications in medicine and in paleooceanography. In animals with skeletons mineralized with calcium, the calcium isotopic composition of soft tissues reflects the relative rate of formation and dissolution of skeletal mineral. In humans, changes in the calcium isotopic composition of urine have been shown to be related to changes in bone mineral balance. When the rate of bone formation exceeds the rate of bone resorption, the $^{44}Ca/^{40}Ca$ ratio in soft tissue rises and vice versa. Because of this relationship, calcium isotopic measurements of urine

or blood may be useful in the early detection of metabolic bone diseases like osteoporosis (Skulan *et al.,* 2007). A similar system exists in seawater, where $^{44}Ca/^{40}Ca$ tends to rise when the rate of removal of Ca^{2+} by mineral precipitation exceeds the input of new calcium into the ocean. In 1997 Skulan and DePaolo presented the first evidence of change in seawater $^{44}Ca/^{40}Ca$ over geologic time, along with a theoretical explanation of these changes. More recent papers have confirmed this observation, demonstrating that seawater Ca^{2+} concentration is not constant, and that the ocean is never in a "steady state" with respect to calcium input and output. This has important climatological implications, as the marine calcium cycle is closely tied to the carbon cycle (Fantle and Depaolo 2007; Griffith *et al.,* 2008).

Many calcium compounds are used in food, as pharmaceuticals, and in medicine, among others. For example, calcium and phosphorus are supplemented in foods through the addition of calcium lactate, calcium diphosphate, and tricalcium phosphate. The last is also used as a polishing agent in toothpaste and in antacids. Calcium lactobionate is a white powder that is used as a suspending agent for pharmaceuticals. In baking, calcium monophosphate is used as a leavening agent. Calcium sulfite is used as a bleach in papermaking and as a disinfectant, calcium silicate is used as a reinforcing agent in rubber, and calcium acetate is a component of liming rosin and is used to make metallic soaps and synthetic resins (Hluchan and Pomerantz, 2005).

2.1.6 Biological and Pathological Roles of Calcium in Biology

Calcium is an essential element needed in large quantities. The Ca^{2+} ion acts as an electrolyte and is vital to the health of the muscular, circulatory, and digestive systems; is indispensable to the building of bone; and supports synthesis and function of blood cells. For example, it regulates the contraction of muscles, nerve conduction, and the clotting of blood. As a result, intra- and

extracellular calcium levels are tightly regulated by the body. Calcium can play this role because the Ca^{2+} ion forms stable coordination complexes with many organic compounds, especially proteins; it also forms compounds with a wide range of solubilities, enabling the formation of skeletons (Hluchan *et al*., 20005).

Calcium ions may be complexed by proteins through binding the carboxyl groups of glutamic acid or aspartic acid residues; through interacting with phosphorylated serine, tyrosine, or threonine residues; or by being chelated by γ-carboxylated amino acid residues. Trypsin, a digestive enzyme, uses the first method; osteocalcin, a bone matrix protein, uses the third. Some other bone matrix proteins such as osteopontin and bone sialoprotein use both the first and the second. Direct activation of enzymes by binding calcium is common; some other enzymes are activated by noncovalent association with direct calcium-binding enzymes. Calcium also binds to the phospholipid layer of the cell membrane, anchoring proteins associated with the cell surface (Hluchan and Pomerantz, 2005). As an example of the wide range of solubility of calcium compounds, monocalcium phosphate is very soluble in water, 85% of extracellular calcium is as dicalcium phosphate with a solubility of 2.0 mM and the hydroxyapatite of bones in an organic matrix is tricalcium phosphate at 100μM (Hluchan and Pomerantz, 2005).

About three-quarters of dietary calcium is from dairy products and grains, the rest being accounted for by vegetables, protein-rich foods, fruits, sugar, fats, and oil. Calcium supplementation is controversial, as the bioavailability of calcium is strongly dependent on the solubility of the salt involved: calcium citrate, malate, and lactate are highly bioavailable while the oxalate is much less so. The intestine absorbs about one-third of calcium eaten as the free ion, and plasma calcium level is then regulated by the kidneys. Parathyroid hormone and vitamin D promote the formation of bone by allowing and enhancing the deposition of calcium ions there,

allowing rapid bone turnover without affecting bone mass or mineral content. When plasma calcium levels fall, cell surface receptors are activated and the secretion of parathyroid hormone occurs; it then proceeds to stimulate the entry of calcium into the plasma pool by taking it from targeted kidney, gut, and bone cells, with the bone-forming action of parathyroid hormone being antagonised by calcitonin, whose secretion increases with increasing plasma calcium levels (Hluchan and Pomerantz, 2005).

Excess intake of calcium may cause hypercalcaemia, but because of the inefficient absorption of calcium by the intestines a more likely cause is excessive vitamin D intake or excessive secretion of parathyroid hormone. It can also occur due to the bone destruction that occurs when tumours metastasise to bone. This results in deposition of calcium salts into the heart, the blood vessels, and the kidneys. Symptoms include anorexia, nausea, vomiting, memory loss, confusion, muscle weakness, increased urination, dehydration, and metabolic bone disease. Chronic hypercalcaemia may lead to soft tissue calcification, which can lead to serious consequences: for example, calcification of the vascular wall can lead to a loss of elasticity and the disruption of laminar blood flow, and thence to plaque rupture and thrombosis. Likewise, inadequate calcium or vitamin D intake results in hypocalcaemia, often caused by inadequate secretion of parathyroid hormone or defective receptors to it in cells. Symptoms include neuromuscular excitability, potentially causing tetany and defects in cardiac conduction (Hluchan and Pomerantz, 2005).

As calcium is heavily involved in bone manufacture, many bone diseases can be traced to problems with the organic matrix or the hydroxyapatite in molecular structure or organisation. For example, osteoporosis is a reduction in mineral content of bone per unit volume, and can be treated by supplementation of calcium, vitamin D, and biphosphates. Calcium supplements may benefit the serum lipids in women who have passed menopause as well as older men; in post-

menopausal women calcium supplementation also appears to be inversely correlated with cardiovascular disease. Inadequate amounts of calcium, vitamin D, or phosphates can lead to the softening of bones, known as osteomalacia (Hluchan and Pomerantz, 2005).

2.2 Zinc

Zinc is a chemical element with symbol Zn and atomic number 30. It is the first element in group 12 of the periodic table. In some respects zinc is chemically similar to magnesium: both elements exhibit only one normal oxidation state (+2), and the Zn^{2+} and Mg^{2+} ions are of similar size. Zinc is the 24th most abundant element in Earth's crust and has five stable isotopes. The most common zinc ore is sphalerite (zinc blende), a zinc sulfide mineral. The largest workable lodes are in Australia, Asia, and the United States. Zinc is refined by froth flotation of the ore, roasting, and final extraction using electricity (electrowinning).

Brass, an alloy of copper and zinc in various proportions, was used as early as the third millennium BC in the Aegean, Iraq, the United Arab Emirates, Kalmykia, Turkmenistan and Georgia, and the second millennium BC in West India, Uzbekistan, Iran, Syria, Iraq, and Israel (Thornton, 2007), Judea (Craddock, 1978). To date, the oldest evidence of pure zinc comes from Zawar, in Rajasthan, as early as the 9th century AD when a distillation process was employed to make pure zinc (Kharakwal and Gurjar, 2006). Alchemists burned zinc in air to form what they called "philosopher's wool" or "white snow".

The element was probably named by the alchemist Paracelsus after the German word *Zinke* (prong, tooth). German chemist Andreas Sigismund Marggraf is credited with discovering pure metallic zinc in 1746. Work by Luigi Galvani and Alessandro Volta uncovered the electrochemical properties of zinc by 1800. Corrosion-resistant zinc plating of iron (hot-dip

galvanizing) is the major application for zinc. Other applications are in electrical batteries, small non-structural castings, and alloys such as brass. A variety of zinc compounds are commonly used, such as zinc carbonate and zinc gluconate (as dietary supplements), zinc chloride (in deodorants), zinc pyrithione (anti-dandruff shampoos), zinc sulfide (in luminescent paints), and zinc methyl or zinc diethyl in the organic laboratory.

Zinc is an essential mineral, including prenatal and postnatal development (Hambidge and Krebs, 2007). Zinc deficiency affects about two billion people in the developing world and is associated with many diseases (Prasad, 2003). In children, deficiency causes growth retardation, delayed sexual maturation, infection susceptibility, and diarrhea (Hambidge and Krebs, 2007). Enzymes with a zinc atom in the reactive center are widespread in biochemistry, such as alcohol dehydrogenase in humans (Maret, 2013). Consumption of excess zinc can cause ataxia, lethargy and copper deficiency.

2.2.1 Physical Properties of Zinc

Zinc is a bluish-white, lustrous, diamagnetic metal, though most common commercial grades of the metal have a dull finish (Wells, 1984). It is somewhat less dense than iron and has a hexagonal crystal-structure, with a distorted form of hexagonal close packing, in which each atom has six nearest neighbors (at 265.9 pm) in its own plane and six others at a greater distance of 290.6 pm (Wells, 1984). Above 210 °C, the metal becomes brittle again and can be pulverized by beating (Scoffern, 1861). Zinc has relatively low melting (419.5 °C) and boiling points (907 °C) (American Galvanizers Association, 2008). The melting point is the lowest of all the d-block metals aside from mercury and cadmium; for this, among other reasons, zinc, cadmium,

and mercury are often not considered to be transition metals like the rest of the d-block metals are (American Galvanizers Association, 2008).

Many alloys contain zinc, including brass. Other metals long known to form binary alloys with zinc are aluminium, antimony, bismuth, gold, iron, lead, mercury, silver, tin, magnesium, cobalt, nickel, tellurium, and sodium (Ingalls, 1902).

A bar of zinc generates a characteristic sound when bent, similar to tin cry.

2.2.2 Isotopes of Zinc

Five isotopes of zinc occur in nature. ^{64}Zn is the most abundant isotope (48.63% natural abundance) (Alejandro, 2008). That isotope has such a long half-life, at 4.3×10^{18} years (Alejandro, 2008). Similarly, ^{70}Zn (0.6%), with a half life of 1.3×10^{16} years is not usually consid ered to be radioactive. The other isotopes found in nature are ^{66}Zn (28%), ^{67}Zn (4%) and ^{68}Zn (1 9%). Several dozen radioisotopes have been characterized. ^{65}Zn, which has a half life of 243.66 d ays, is the least active radioisotope, followed by ^{72}Zn with a half-life of 46.5 hours (Alejandro, 2008). Zinc has 10 nuclear isomers. ^{69m}Zn has the longest half-life, 13.76 h (Alejandro, 2008). The superscript *m* indicates a metastable isotope. The nucleus of a metastable isotope is in an excited state and will return to the ground state by emitting a photon in the form of a gamma ray. ^{61}Zn has three excited metastable states and 73 Zn has two (Audi *et al*., 2003). The isotopes ^{65}Zn, ^{71}Zn, ^{77}Zn and ^{78}Zn each have only one excited metastable state (Alejandro, 2008).

The most common decay mode of a radioisotope of zinc with a mass number lower than 66 is electron capture. The decay product resulting from electron capture is an isotope of copper (Alejandro, 2008).

n30Zn+ e → n29Cu

The most common decay mode of a radioisotope of zinc with mass number higher than 66 is beta decay (β^-), which produces an isotope of gallium (Alejandro, 2008).

n30Zn → n31Ga+ e +νe

2.2.3 Compounds and Chemical Reactivity of Zinc

Zinc has an electron configuration of $[Ar]3d^{10}4s^2$ and is a member of the group 12 of the periodic table. It is a moderately reactive metal and strong reducing agent (Hinds and Iredelle, 1908). The surface of the pure metal tarnishes quickly, eventually forming a protective passivating layer of the basic zinc carbo-nate, $Zn_5(OH)\ 6(CO_3)_2$, by reaction with atmospheric carbon dioxide (Porter, 1994). This layer helps prevent further reaction with air and water.

Zinc burns in air with a bright bluish-green flame, giving off fumes of zinc oxide (Holleman *et al.,* 1985). Zinc reacts readily with acids, alkalis and other non-metals (Hinds and Iredelle, 1908). Extremely pure zinc reacts only slowly at room temperature with acids (Holleman *et al.,* 1985). Strong acids, such as hydrochloric or sulfuric acid, can remove the passivating layer and subsequent reaction with water releases hydrogen gas (Holleman *et al.,* 1985).

The chemistry of zinc is dominated by the +2 oxidation state. When compounds in this oxidation state are formed, the outer shell *s* electrons are lost, yielding a bare zinc ion with the electronic configuration $[Ar]3d^{10}$ (Ritchie, 2004). In aqueous solution an octahedral co mplex, $[Zn(H_2O)_6]_2^+$ is the predominant species (Burgess, 1978). The volatilization of zinc in combination with zinc chloride at temperatures above 285 °C indicates the formation of Zn_2Cl_2, a zinc compound with a +1 oxidation state (Holleman *et al.,* 1985). No compounds of zinc in

oxidation states other than +1 or +2 are known (Brady *et al*., 1983). Calculations indicate that a zinc compound with the oxidation state of +4 is unlikely to exist (Kaupp *et al.,* 1994).

Zinc chemistry is similar to the chemistry of the late first-row transition metals, nickel and copper, though it has a filled d-shell and compounds are diamagnetic and mostly colorless (Holleman *et al.,* 1985). The ionic radii of zinc and magnesium happen to be nearly identical. Because of this some of the equivalent salts have the same crystal structure (Holleman *et al.,* 1985), and in other circumstances where ionic radius is a determining factor, the chemistry of zinc has much in common with that of magnesium (Holleman *et al.,* 1985). In other respects, there is little similarity with the late first-row transition metals. Zinc tends to form bonds with a greater degree of covalency and much more stable complexes with N- and S- donors (Holleman *et al.,* 1985). Complexes of zinc are mostly 4- or 6- coordinate although 5-coordinate complexes are known (Holleman *et al.,* 1985).

2.2.4 Zinc(I) Compound

Zinc(I) compounds are rare and need bulky ligands to stabilize the low oxidation state. Most zinc(I) compounds contain formally the $[Zn_2]^{2+}$ core, which is analogous to the $[Hg_2]^{2+}$ dimeric cation present in mercury(I) compounds. The diamagnetic nature of the ion confirms its dimeric structure. The first zinc(I) compound containing the Zn–Zn bond, $(\eta^5\text{-}C_5Me_5)_2Zn_2$, is also the first dimetallocene. The $[Zn_2]^{2+}$ ion rapidly disproportionates into zinc metal and zinc(II), and has been obtained only a yellow glass only by cooling a solution of metallic zinc in molten $ZnCl_2$ (Housecroft and Sharpe 2008).

Figure 2.2: Zinc(II) Compound

2.2.5 Zinc(II) Compound

Binary compounds of zinc are known for most of the metalloids and all the nonmetals except the noble gases. The oxide ZnO is a white powder that is nearly insoluble in neutral aqueous solutions, but is amphoteric, dissolving in both strong basic and acidic solutions (Holleman *et al.,* 1985*).* The other chalcogenides (ZnS, ZnSe, and ZnTe) have varied applications in electronics and optics (Porter, 1994). Pnictogenides (Zn 3N2, Zn3P2, Zn3As2 and Zn3Sb2), (Gr olier*,* 1994). The peroxide (ZnO_2), the hydride (ZnH_2), and the carbide (ZnC_2) are also known (Shulzhenko *et al.,* 2000). Of the four halides, ZnF_2 has the most ionic character, while t he others ($ZnCl_2$, $ZnBr_2$, and ZnI_2) have relatively low melting points and are considered to have more covalent character (Shulzhenko *et al.,* 2000).

In weak basic solutions containing Zn2+ ions, the hydroxide $Zn(OH)_2$ forms as a white precipitate. In stronger alkaline solutions, this hydroxide is dissolved to form zincates ($[Zn(OH)_4]2-$) (Holleman *et al.,* 1985).

The nitrate $Zn(NO_3)_2$, chlorate $Zn(ClO_3)_2$, sulfate ZnSO4, phosphate $Zn3(PO_4)_2$, molybdate $ZnMoO_4$, cyanide $Zn(CN)_2$, arsenite $Zn(AsO_2)_2$, arsenate $Zn(AsO_4)2{\cdot}8H_2O$ and the chromate $ZnCrO_4$ (one of the few colored zinc compounds) are a few examples of other common inorganic compounds of zinc (Rasmussen and Heilmann, 1990; Perry*,*1995). One of the simplest examples of an organic compound of zinc is the acetate ($Zn(O_2CCH_3)_2$). Organozinc compounds are those that contain zinc–carbon covalent bonds. Diethylzinc ($C2H_5$)2Zn) is a reagent in synthetic chemistry. It was first reported in 1848 from the reaction of zinc and ethyl iodide, and was the first compound known to contain a metal–carbon sigma bond (Frankland*,* 1850).

2.2.6 Test for Zinc

Cobalticyanide paper (Rinnmann's test for Zn) can be used as a chemical indicator for zinc. 4 g of $K_3Co(CN)_6$ and 1 g of $KClO_3$ is dissolved on 100 ml of water. Paper is dipped in the solution and dried at 100 °C. One drop of the sample is dropped onto the dry paper and heated. A green disc indicates the presence of zinc (Lide, 1998).

2.2.7 Early Studies and Naming of Zinc

Zinc was distinctly recognized as a metal under the designation of *Yasada* or Jasada in the medical Lexicon ascribed to the Hindu king Madanapala (of Taka dynasty) and written about the year 1374 (Ray and Chandra 1903). Smelting and extraction of impure zinc by reducing calamine with wool and other organic substances was accomplished in the 13th century in India (Habashi, 2009). The Chinese did not learn of the technique until the 17th century (Habashi, 2009). Alchemists burned zinc metal in air and collected the resulting zinc oxide on a condenser. Some alchemists called this zinc oxide *lana philosophica*, Latin for "philosopher's wool", because it collected in wooly tufts, whereas others thought it looked like white snow and named it *nix album* (Arny, 1917).

The name of the metal was probably first documented by Paracelsus, a Swiss-born German alchemist, who referred to the metal as "zincum" or "zinken" in his book *Liber Mineralium II*, in the 16th century (Habashi, 2009; Hoover, 2003). The word is probably derived from the German *zinke*, and supposedly meant "tooth-like, pointed or jagged" (metallic zinc crystals have a needle-like appearance) (Gerhartz *et al.,* 1996). Zink could also imply "tin-like" because of its relation to German *zinn* meaning tin (Skeat, 2005) Yet another possibility is that the word is derived from the Persian word *seng* meaning stone (Fathi, 1997). German metallurgist Andreas Libavius

received a quantity of what he called "calay" of Malabar from a cargo ship captured from the Portuguese in 1596 (Lach, 1994). Libavius described the properties of the sample, which may have been zinc. Zinc was regularly imported to Europe from the Orient in the 17th and early 18th centuries (Habashi, 2009).

2.2.8 Isolation of Zinc

Metallic zinc was isolated in India by 1300 AD (Vaughan, 1897; Castellani, 2014; Habib, 2011), much earlier than in the West. Before it was isolated in Europe, it was imported from India in about 1600 CE (Jenkins, 1945) Postlewayt's *Universal Dictionary*, a contemporary source giving technological information in Europe, did not mention zinc before 1751 but the element was studied before then (Craddock *et al.,* 1983; Willies *et al.,* 1984).

In Britain, John Lane is said to have carried out experiments to smelt zinc, probably at Landore, prior to his bankruptcy in 1726 (Roberts, 1951). In 1738 in Great Britain, William Champion patented a process to extract zinc from calamine in a vertical retort style smelter (Comyns, 2007). His technique resembled that used at Zawar zinc mines in Rajasthan, but no evidence suggests he visited the Orient (Jenkins, 1945). Champion's process was used through 1851 (Habashi, 2009).

German chemist Andreas Marggraf normally gets credit for discovering pure metallic zinc, even though Swedish chemist Anton von Swab had distilled zinc from calamine four years previously (Habashi, 2009).

Prior to this, only calamine could be used to produce zinc. In 1798, Johann Christian Ruberg improved on the smelting process by building the first horizontal retort smelter (Gray, 2005). Jean-Jacques Daniel Dony built a different kind of horizontal zinc smelter in Belgium that processed even more zinc (Habashi, 2009). Italian doctor Luigi Galvani discovered in 1780 that

connecting the spinal cord of a freshly dissected frog to an iron rail attached by a brass hook caused the frog's leg to twitch (Warren, 2000).

Galvani's friend, Alessandro Volta, continued researching the effect and invented the Voltaic pile in 1800 (Warren, 2000). The basic unit of Volta's pile was a simplified galvanic cell, made of plates of copper and zinc separated by an electrolyte and connected by a conductor externally. The units were stacked in series to make the Voltaic cell, which produced electricity by directing electrons from the zinc to the copper and allowing the zinc to corrode (Warren, 2000).

2.2.9 Production of Zinc

Zinc is the fourth most common metal in use, trailing only iron, aluminium, and copper with an annual production of about 13 million tonnes (Tolcin, 2015). The world's largest zinc producer is Nyrstar, a merger of the Australian OZ Minerals and the Belgian Umicore (Attwood, 2006). About 70% of the world's zinc originates from mining, while the remaining 30% comes from recycling secondary zinc (Attwood, 2006). Commercially pure zinc is known as Special High Grade, often abbreviated *SHG*, and is 99.995% pure (Attwood, 2006).

Worldwide, 95% of new zinc is mined from sulfidic ore deposits, in which sphalerite (ZnS) is nearly always mixed with the sulfides of copper, lead and iron (Porter, 1991). Zinc mines are scattered throughout the world, with the main areas being China, Australia, and Peru. China produced 38% of the global zinc output in 2014 (Tolcin, 2015).

Zinc metal is produced using extractive metallurgy (Rosenqvist, 1922), The ore is finely ground, then put through froth flotation to separate minerals from gangue (on the property of hydrophobicity), to get a zinc sulfide ore concentrate (Rosenqvist, 1922) consisting of about 50% zinc, 32% sulfur, 13% iron, and 5% SiO2 (Rosenqvist, 1922)

Roasting converts the zinc sulfide concentrate to zinc oxide: (Porter, 1991)

$$2ZnS + 3O_2 \rightarrow 2ZnO + 2SO_2$$

The sulfur dioxide is used for the production of sulfuric acid, which is necessary for the leaching process. If deposits of zinc carbonate, zinc silicate, or zinc spinel (like the Skorpion Deposit in Namibia) are used for zinc production, the roasting can be omitted (Borg *et al.,* 2003).

For further processing two basic methods are used: pyrometallurgy or electrowinning. Pyrometallurgy reduces zinc oxide with carbon or carbon monoxide at 950 °C (1,740 °F) into the metal, which is distilled as zinc vapor to separate it from other metals, which are not volatile at those temperatures (Bodsworth, 1994). The zinc vapor is collected in a condenser (Porter, 1991). The equations below describe this process: (Porter, 1991).

$$2ZnO + C \rightarrow 2Zn + CO_2$$

$$ZnO + CO \rightarrow Zn + CO_2$$

In electrowinning, zinc is leached from the ore concentrate by sulfuric acid: (Gupta and Mukherjee 1990).

$$ZnO + H_2SO4 \rightarrow ZnSO_4 + H_2O$$

Finally, the zinc is reduced by electrolysis (Porter, 1991).

$$2ZnSO_4 + 2H_2O \rightarrow 2Zn + 2H_2SO_4 + O_2$$

The sulfuric acid is regenerated and recycled to the leaching step.

When galvanised feedstock is fed to an electric arc furnace, the zinc is recovered from the dust by a number of processes, predominately the Waelz process (90% as of 2014) (Antrekowitsch *et al.,* 2014).

2.2.10 Environmental Impact of Zinc

Refinement of sulfidic zinc ores produces large volumes of sulfur dioxide and cadmium vapor. Smelter slag and other residues contain significant quantities of metals. About 1.1 million tonnes of metallic zinc and 130 thousand tonnes of lead were mined and smelted in the Belgian towns of La Calamine and Plombières between 1806 and 1882 (Kucha *et al.,* 1996). The dumps of the past mining operations leach zinc and cadmium, and the sediments of the Geul River contain non-trivial amounts of metals (Kucha *et al.,* 1996). About two thousand years ago, emissions of zinc from mining and smelting totaled 10 thousand tonnes a year. After increasing 10-fold from 1850, zinc emissions peaked at 3.4 million tonnes per year in the 1980s and declined to 2.7 million tonnes in the 1990s, although a 2005 study of the Arctic troposphere found that the concentrations there did not reflect the decline. Anthropogenic and natural emissions occur at a ratio of 20 to 1 (Broadley *et al.,* 2007).

Historically responsible for high metal levels in the Derwent River (Change, 2009), the zinc works at Lutana is the largest exporter in Tasmania, generating 2.5% of the state's GDP, and producing more than 250 000 tonnes of zinc per year (Change, 2009).

2.2.11 Other Industrial Uses of Zinc

Roughly one quarter of all zinc output in the United States in 2009 was consumed in zinc compounds and many of which are used industrially (Xie *et al.,* 2013). Zinc oxide is widely used as a white pigment in paints and as a catalyst in the manufacture of rubber to disburse heat. The semiconductor properties of zinc oxide make it useful in varistors and photocopying products (Zhang, 1996). The zinc zinc-oxide cycle is a two step thermochemical process based on zinc and zinc oxide for hydrogen production (Weimer, 2006).

Zinc chloride is often added to lumber as a fire retardant and sometimes as a wood preservative (Blew, 1953). It is used in the manufacture of other chemicals (Blew, 1953). Zinc methyl ($Zn(CH_3)_2$) is used in a number of organic syntheses (Frankland, 1849). Zinc sulfide (ZnS) is used in luminescent pigments such as on the hands of clocks, X-ray and television screens, and luminous paints (Paschotta, 2008). Crystals of ZnS are used in lasers that operate in the mid-infrared part of the spectrum (Konstantinou, 2004). Zinc sulfate is a chemical in dyes and pigments (Blew, 1953). Zinc pyrithione is used in antifouling paints (Konstantinou and Albanis 2004).

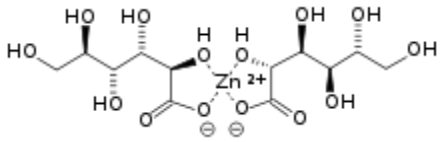
Zinc powder is sometimes used as a propellant in model rockets (Boudreaux, 2008). When a compressed mixture of 70% zinc and 30% sulfur powder is ignited there is a violent chemical reaction (Boudreaux, 2008). This produces zinc sulfide, together with large amounts of hot gas, heat, and light (Boudreaux, 2008).

A jacket of isotopically enriched ^{64}Zn would be irradiated by the intense high-energy neutron flux from an exploding thermonuclear weapon, forming a large amount of 65Zn significantly increasing the radioactivity of the weapon's fallout (Win and Tin, 2003). Such a weapon is not known to have ever been built, tested, or used (Win and Tin, 2003). ^{65}Zn is used as a tracer to study how alloys that contain zinc wear out, or the path and the role of zinc in organisms (David, 1999).

Zinc dithiocarbamate complexes are used as agricultural fungicides; these include Zineb, Metiram, Propineb and Ziram (Wiley-Vch, 2007). Zinc naphthenate is used as wood preservative (Walker, 2006).

2.2.12 Dietary Supplement of Zinc

GNC zinc 50 mg tablets. The amount exceeds what is deemed the safe upper limit in the United States (40 mg) and European Union (25 mg)



Zinc gluconate is one compound used for the delivery of zinc as a dietary supplement. In most single-tablet, over-the-counter, daily vitamin and mineral supplements, zinc is included in such forms as zinc oxide, zinc acetate, or zinc gluconate (Wolfgang, 2013). Zinc is generally considered to be an antioxidant. However, it is redox inert and thus can serve such a function only indirectly (Wolfgang, 2013).

Zinc deficiency has been associated with major depressive disorder (MDD), and zinc supplements may be an effective treatment (Swardfager *et al.,* 2013).

Zinc serves as a simple, inexpensive, and critical tool for treating diarrheal episodes among children in the developing world. Zinc becomes depleted in the body during diarrhea, but recent studies suggest that replenishing zinc with a 10- to 14-day course of treatment can reduce the duration and severity of diarrheal episodes and may also prevent future episodes for as long as three months (Bhutta *et al.,* 2000).

A Cochrane review stated that people taking zinc supplement may be less likely to progress to age-related macular degeneration (EvIains and Lawrenson, 2017).

Gastroenteritis is strongly attenuated by ingestion of zinc, possibly by direct antimicrobial action of the ions in the gastrointestinal tract, or by the absorption of the zinc and re-release from immune cells (all granulocytes secrete zinc), or both (Aydemir *et al*., 2006; Valko, 2005).

In 2011, researchers reported that adding large amounts of zinc to a urine sample masked detection of drugs. The researchers did not test whether orally consuming a zinc dietary supplement could have the same effect (Venkatratnam and Nathan, 2011).

2.2.13 Common Cold

Zinc supplements (frequently zinc acetate or zinc gluconate lozenges) are a group of dietary supplements that are commonly used for the treatment of the common cold (Singh, 2013). The use of zinc supplements at doses in excess of 75 mg/day within 24 hours of the onset of symptoms has been shown to reduce the duration of cold symptoms by about 1 day (Singh, 2013). Due to a lack of data, there is insufficient evidence to determine whether the preventative use of zinc supplements reduces the likelihood of contracting a cold (Singh, 2013). Adverse effects with zinc supplements by mouth include bad taste and nausea (Singh, 2013).

The human rhinovirus – the most common viral pathogen in humans – is the predominant cause of the common cold (Roldán, 2003). The hypothesized mechanism of action by which zinc reduces the severity and/or duration of cold symptoms is the suppression of nasal inflammation and the direct inhibition of rhinoviral receptor binding and rhinoviral replication in the nasal mucosa (Singh, 2013).

2.2.14 Biological Role of Zinc

Zinc is an essential trace element for humans (Maret, 2013; Prakash, 2015; Cherasse, 2017). and other animals (Prasad, 2008), for plants (Broadley *et al.,* 2007) and for microorganisms. Zinc is required for the function of over 300 enzymes and 1000 transcription factors (Cherasse and Urade, 2017), and is stored and transferred in metallothioneins (Plum, 2010). It is the second

most abundant trace metal in humans after iron and it is the only metal which appears in all enzyme classes (Broadley *et al.,* 2007; Cherasse *et al.,* 2017).

In proteins, zinc ions are often coordinated to the amino acid side chains of aspartic acid, glutamic acid, cysteine and histidine. The theoretical and computational description of this zinc binding in proteins (as well as that of other transition metals) is difficult (Brandt *et al.,* 2009).

Roughly 2–4 grams of zinc (Rink and Gabriel, 2000). are distributed throughout the human body. Most zinc is in the brain, muscle, bones, kidney, and liver, with the highest concentrations in the prostate and parts of the eye (Wapnir, 1990). Semen is particularly rich in zinc, a key factor in prostate gland function and reproductive organ growth (Berdanier *et al.,* 2007).

In humans, the biological roles of zinc are ubiquitous (Prakash *et al.,* 2015). It also regulates apoptosis. A 2006 study estimated that about 10% of human proteins 2800 potentially bind zinc, in addition to hundreds more that transport and traffic zinc; a similar *in silico* study in the plant *Arabidopsis thaliana* found 2367 zinc-related proteins (Broadley *et al.,* 2007).

In the brain, zinc is stored in specific synaptic vesicles by glutamatergic neurons and can modulate neuronal excitability (Prakash *et al.,* 2015; Cherasse and Urade 2017; Prasad, 2008). It plays a key role in synaptic plasticity and so in learning (Prakash *et al.,* 2015; Nakashima and Dyck, 2009*)*. Zinc homeostasis also plays a critical role in the functional regulation of the central nervous system (Prakash *et al.,* 2015*;* Cherasse and Urade, 2017). Dysregulation of zinc homeostasis in the central nervous system that results in excessive synaptic zinc concentrations is believed to induce neurotoxicity through mitochondrial oxidative stress (e.g., by disrupting certain enzymes involved in the electron transport chain, including complex I, complex III, and α-ketoglutarate dehydrogenase), the dysregulation of calcium homeostasis, glutamatergic

neuronal excitotoxicity, and interference with intraneuronal signal transduction (Prakash *et al.,* 2015; Tyszka *et al.,* 2014).

2.2.15 Enzymes of Zinc

Zinc is an efficient Lewis acid, making it a useful catalytic agent in hydroxylation and other enzymatic reactions (Stipanuk, 2006). The metal also has a flexible coordination geometry, which allows proteins using it to rapidly shift conformations to perform biological reactions (Stipanuk, 2006). Two examples of zinc-containing enzymes are carbonic anhydrase and carboxypeptidase, which are vital to the processes of carbon dioxide (CO_2) regulation and digestion of proteins, respectively (Stipanuk, 2006).

In vertebrate blood, carbonic anhydrase converts CO_2 into bicarbonate and the same enzyme transforms the bicarbonate back into CO_2 for exhalation through the lungs (Kohen *et al.,* 2006). The non-related β-carbonic anhydrase is required in plants for leaf formation, the synthesis of indole acetic acid (auxin) and alcoholic fermentation (Gadallah, 2000).

2.2.16 Other Proteins of Zinc

In blood plasma, zinc is bound to and transported by albumin (60%, low-affinity) and transferrin (10%) (Wapnir, 1990). Because transferrin also transports iron, excessive iron reduces zinc absorption, and vice versa. A similar antagonism exists with copper (Whitney *et al.,* 2005). Cells in the salivary gland, prostate, immune system, and intestine use zinc signaling to communicate with other cells (Hershfinkel *et al.,* 2007).

Metallothionein in intestinal cells is capable of adjusting absorption of zinc by 15–40% (Blake, 2007). However, inadequate or excessive zinc intake can be harmful; excess zinc particularly impairs copper absorption because metallothionein absorbs both metals (Fosmire, 1990).

The human dopamine transporter contains a high affinity extracellular zinc binding site which, upon zinc binding, inhibits dopamine reuptake and amplifies amphetamine-induced dopamine efflux *in vitro* (Krause, 2008; Sulzer, 2011; Scholze *et al.,* 2002). The human serotonin transporter and norepinephrine transporter do not contain zinc binding sites (Scholze *et al.,* 2002).

2.2.17 Dietary Intake of Zinc

Animal products (meat, fish, shellfish, fowl, eggs, dairy) contain zinc. The concentration of zinc in plants varies with the level in the soil. With adequate zinc in the soil, the food plants that contain the most zinc are wheat (germ and bran) and various seeds (sesame, poppy, alfalfa, celery, mustard) (Allen, 1998). Zinc is also found in beans, nuts, almonds, whole grains, pumpkin seeds, sunflower seeds and blackcurrant (Allen, 1998). Plant phytates interfere with zinc absorption, so people consuming a vegetarian or vegan diet may need to increase zinc intake. Other sources include fortified food and dietary supplements in various forms. A 1998 review concluded that zinc oxide, one of the most common supplements in the United States, and zinc carbonate are nearly insoluble and poorly absorbed in the body (Allen, 1998). This review cited studies that found lower plasma zinc concentrations in the subjects who consumed zinc oxide and zinc carbonate than in those who took zinc acetate and sulfate salts (Allen, 1998). For fortification, however, a 2003 review recommended cereals (containing zinc oxide) as a cheap, stable source that is as easily absorbed as the more expensive forms (Rosado, 2003). A 2005 study found that various compounds of zinc, including oxide and sulfate, did not show

statistically significant differences in absorption when added as fortificants to maize tortillas (Hotz *et al.,* 2005).

2.2.18 Deficiency of Zinc

Zinc deficiency is usually due to insufficient dietary intake, but can be associated with malabsorption, acrodermatitis enteropathica, chronic liver disease, chronic renal disease, sickle cell disease, diabetes, malignancy, and other chronic illnesses (Prasad*,* 2003*)*. Groups at risk for zinc deficiency include the elderly, children in developing countries, and those with renal dysfunction.

In the United States, a federal survey of food consumption determined that for women and men over the age of 19, average consumption was 9.7 and 14.2 mg/day, respectively. For women, 17% consumed less than the EAR, for men 11%. The percentages below EAR increased with age (Moshfegh *et al.,* 2005). The most recent published update of the survey (NHANES 2013–2014) reported lower averages – 9.3 and 13.2 mg/day – again with intake decreasing with age (Moshfegh *et al.,* 2005).

Symptoms of mild zinc deficiency are diverse (Rink and Gabriel, 2000). Clinical outcomes include depressed growth, diarrhea, impotence and delayed sexual maturation, alopecia, eye and skin lesions, impaired appetite, altered cognition, impaired host defense properties, defects in carbohydrate utilization, and reproductive teratogenesis and mild zinc deficiency depresses immunity (Ibs and Rink 2003), although excessive zinc does also (Rink and Gabriel, 2000).

Western vegetarians and vegans do not experience any from overt zinc deficiency than meat-eaters (Freeland-Graves *et al.,* 1980). Major plant sources of zinc include cooked dried beans, sea vegetables, fortified cereals, soy foods, nuts, peas, and seeds (Freeland-Graves *et al.,* 1980).

However, phytates in many whole-grains and fibers may interfere with zinc absorption and marginal zinc intake has poorly understood effects. The zinc chelator phytate, found in seeds and cereal bran, can contribute to zinc malabsorption (Prasad, 2003). Some evidence suggests that more than the US RDA (15 mg) of zinc daily may be needed in those whose diet is high in phytates, such as some vegetarians (Freeland-Graves *et al.,* 1980). These considerations must be balanced against the paucity of adequate zinc biomarkers, and the most widely used indicator, plasma zinc, has poor sensitivity and specificity (Hambidge, 2003). Diagnosing zinc deficiency is a persistent challenge (Hambidge and Krebs, 2007).

The World Health Organization advocates zinc supplementation for severe malnutrition and diarrhea (W.H.O, 2007). Zinc supplements help prevent disease and reduce mortality, especially among children with low birth weight or stunted growth (W.H.O, 2007). However, zinc supplements should not be administered alone, because many in the developing world have several deficiencies, and zinc interacts with other micronutrients (Shrimpton *et al.,* 2005).

2.2.19 Zinc Toxicity

Although zinc is an essential requirement for good health, excess zinc can be harmful. Excessive absorption of zinc suppresses copper and iron absorption (Fosmire, 1990). The free zinc ion in solution is highly toxic to plants, invertebrates, and even vertebrate fish (Eisler, 1993). The Free Ion Activity Model is well-established in the literature, and shows that just micromolar amounts of the free ion kills some organisms. A recent example showed 6 micromolar killing 93% of all *Daphnia* in water (Muyssen, *et al.,* 2006).

The free zinc ion is a powerful Lewis acid up to the point of being corrosive. Stomach acid contains hydrochloric acid, in which metallic zinc dissolves readily to give corrosive zinc

chloride. Swallowing a post-1982 American one cent piece (97.5% zinc) can cause damage to the stomach lining through the high solubility of the zinc ion in the acidic stomach (Bothwell *et al.,* 2003).

Evidence shows that people taking 100-300mg of zinc daily may suffer induced copper deficiency. A 2007 trial observed that elderly men taking 80 mg daily were hospitalized for urinary complications more often than those taking a placebo (Johnson *et al*., 2007). Levels of 100-300mg may interfere with the utilization of copper and iron or adversely affect cholesterol (Fosmire, 1990). A condition called the zinc shakes or "zinc chills" can be induced by inhalation of zinc fumes while brazing or welding galvanized materials (Paschotta, 2008). Zinc is a common ingredient of denture cream which may contain between 17 and 38 mg of zinc per gram. Disability and even deaths from excessive use of these products have been claimed (Oxford and Öberg 1985).

The U.S. Food and Drug Administration (FDA) states that zinc damages nerve receptors in the nose, causing anosmia. Reports of anosmia were also observed in the 1930s when zinc preparations were used in a failed attempt to prevent polio infections (Oxford and Öberg 1985).

Recent research suggests that the topical antimicrobial zinc pyrithione is a potent heat shock response inducer that may impair genomic integrity with induction of PARP-dependent energy crisis in cultured human keratinocytes and melanocytes (Lamore *et al.,* 2010).

2.2.20 Poisonous Effect of Zinc

In 1982, the US Mint began minting pennies coated in copper but containing primarily zinc. Zinc pennies pose a risk of zinc toxicosis, which can be fatal. One reported case of chronic ingestion of 425 pennies (over 1 kg of zinc) resulted in death due to gastrointestinal bacterial and fungal

sepsis. Another patient who ingested 12 grams of zinc showed only lethargy and ataxia (gross lack of coordination of muscle movements) (Bennett *et al.,* 1997). Several other cases have been reported of humans suffering zinc intoxication by the ingestion of zinc coins (Bennett *et al.,* 1997; Fernbach and Tucker 1986).

Pennies and other small coins are sometimes ingested by dogs, requiring veterinary removal of the foreign objects. The zinc content of some coins can cause zinc toxicity, commonly fatal in dogs through severe hemolytic anemia and liver or kidney damage; vomiting and diarrhea are possible symptoms (Stowe *et al.,* 1978). Zinc is highly toxic in parrots and poisoning can often be fatal (Reece *et al.,* 1986). The consumption of fruit juices stored in galvanized cans has resulted in mass parrot poisonings with zinc (Ayuk and Gittoes 2014).

2.3 Copper

Copper is a soft, malleable, and ductile metal with very high thermal and electrical conductivity. It is chemical element with symbol **Cu** (from Latin: *cuprum*) and atomic number 29. A freshly exposed surface of pure copper has a reddish-orange color. Copper is used as a conductor of heat and electricity, as a constituent of various metal alloys, such as sterling silver used in jewelry, cupronickel used to make marine hardware and coins, and constantan used in strain gauges and thermocouples for temperature measurement and is also the building material (McHenry*,* 1992).

Copper is one of the few metals that occur in nature in directly usable metallic form (native metals) as opposed to needing extraction from an ore. This led to very early human use, from c. 8000 BC. It was the first metal to be smelted from its ore, c. 5000 BC, the first metal to be cast

into a shape in a mold, c. 4000 BC and the first metal to be purposefully alloyed with another metal, tin, to create bronze, c. 3500 BC (McHenry, 1992).

The commonly encountered compounds are copper(II) salts, which often impart blue or green colors to such minerals as azurite, malachite, and turquoise, and have been used widely and historically as pigments. Copper used in buildings, usually for roofing, oxidizes to form a green verdigris (or patina). Copper is sometimes used in decorative art, both in its elemental metal form and in compounds as pigments. Copper compounds are used as bacteriostatic agents, fungicides, and wood preservatives.

Copper is essential to all living organisms as a trace dietary mineral because it is a key constituent of the respiratory enzyme complex cytochrome c oxidase. In molluscs and crustaceans, copper is a constituent of the blood pigment hemocyanin, replaced by the iron-complexed hemoglobin in fish and other vertebrates. In humans, copper is found mainly in the liver, muscle, and bone (Johnson *et al.,* 2008).

2.3.1 Physical Characteristics of Copper

A copper disc (99.95% pure) made by continuous casting; etched to reveal crystallites. Copper just above its melting point keeps its pink luster color when enough light outshines the orange incandescence color Copper, silver, and gold are in group 11 of the periodic table; these three metals have one s-orbital electron on top of a filled d-electron shell and are characterized by high ductility, and electrical and thermal conductivity. The filled d-shells in these elements contribute little to interatomic interactions, which are dominated by the s-electrons through metallic bonds. Unlike metals with incomplete d-shells, metallic bonds in copper are lacking a covalent character and are relatively weak. This observation explains the low hardness and high ductility of single

crystals of copper (George *et al*., 1992). At the macroscopic scale, introduction of extended defects to the crystal lattice, such as grain boundaries, hinders flow of the material under applied stress, thereby increasing its hardness. For this reason, copper is usually supplied in a fine-grained polycrystalline form, which has greater strength than monocrystalline forms (Smith *et al.,* 2003).

The softness of copper partly explains its high electrical conductivity (59.6×10^6 S/m) and high thermal conductivity, second highest (second only to silver) among pure metals at room temperature (Hammond, 2004). This is because the resistivity to electron transport in metals at room temperature originates primarily from scattering of electrons on thermal vibrations of the lattice, which are relatively weak in a soft metal (George and Edmund 1992).

Copper is one of a few metallic elements with a natural color other than gray or silver (Chambers and Chambers 1884).

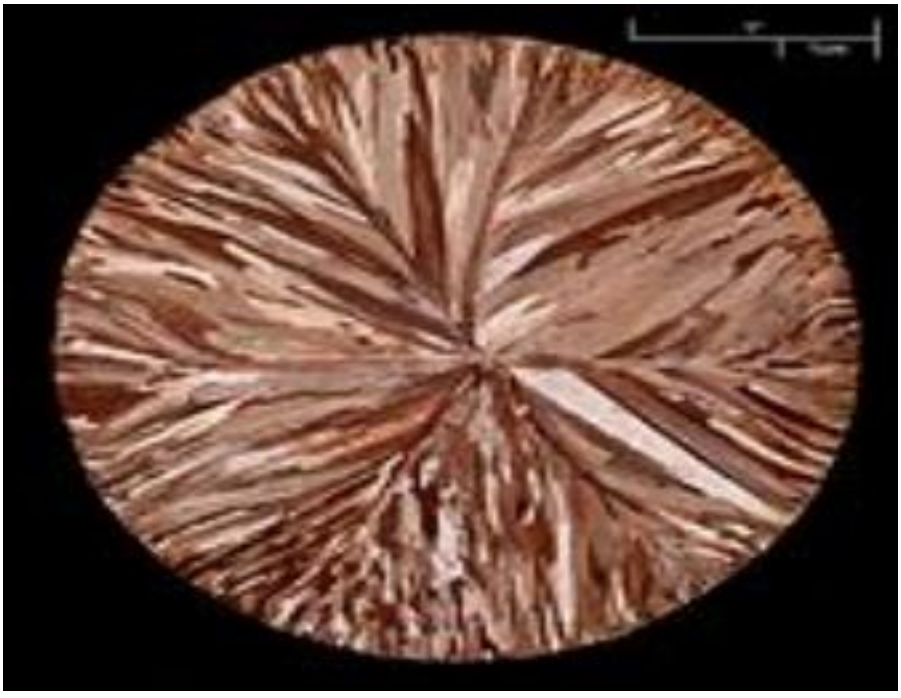


Figure 2.3: Copper

2.3.2 Isotopes of Copper

There are 29 isotopes of copper. ^{63}Cu and ^{65}Cu are stable, with ^{63}Cu comprising approximately 69% of naturally occurring copper; both have a spin of ½ (Audi *et al.,* 2003). The other isotopes are radioactive, with the most stable being ^{67}Cu with a half-life of 61.83 hours (Audi *et al.,* 2003).

2.3.3 Occurrence of Copper

2.5 inches (6.4 cm) long Copper is produced in massive stars (Romano and Matteucci 2007) and is present in the Earth's crust in a proportion of about 50 parts per million (ppm) (Emsley,

2003). It occurs as native copper, in the copper sulfides chalcopyrite and chalcocite, in the copper carbonates azurite and malachite, and in the copper(I) oxide mineral cuprite (Hammond, 2004). The largest mass of elemental copper discovered weighed 420 tonnes and was found in 1857 on the Keweenaw Peninsula in Michigan, US (Emsley, 2003). Native copper is a polycrystal, with the largest single crystal ever described measuring 4.4×3.2×3.2 cm (Rickwood, 1981).

2.3.4 Production of Copper

Chuquicamata, in Chile, is one of the world's largest open pit copper mines Most copper is mined or extracted as copper sulfides from large open pit mines in porphyry copper deposits that contain 0.4 to 1.0% copper. Sites include Chuquicamata, in Chile, Bingham Canyon Mine, in Utah, United States, and El Chino Mine, in New Mexico, United States. According to the British Geological Survey, in 2005, Chile was the top producer of copper with at least one-third of the world share followed by the United States, Indonesia and Peru (Hammond, 2004). Copper can also be recovered through the in-situ leach process. Several sites in the state of Arizona are considered prime candidates for this method (Randazzo, 2011). The amount of copper in use is increasing and the quantity available is barely sufficient to allow all countries to reach developed world levels of usage (Gordon *et al.,* 2006).

2.3.5 Reservation of Copper

Copper has been in use at least 10,000 years, but more than 95% of all copper ever mined and smelted has been extracted since 1900 (Leonard, 2006), and more than half was extracted in the last 24 years. As with many natural resources, the total amount of copper on Earth is vast, with

around 10^{14} tons in the top kilometer of Earth's crust, which is about 5 million years' worth at the current rate of extraction. However, only a tiny fraction of these reserves is economically viable with present-day prices and technologies. Estimates of copper reserves available for mining vary from 25 to 60 years, depending on core assumptions such as the growth rate (Brown, 2006). Recycling is a major source of copper in the modern world (Leonard, 2006). Because of these and other factors, the future of copper production and supply is the subject of much debate, including the concept of peak copper, analogous to peak oil.

The price of copper has historically been unstable (Schmitz, 1986), and its price increased from the 60 - year low of US$0.60/lb (US$1.32/kg) in June 1999 to $3.75 per pound ($8.27/kg) in May 2006. It dropped to $2.40/lb ($5.29/kg) in February 2007, In February 2009, weakening global demand and a steep fall in commodity prices since the previous year's highs left copper prices at $1.51/lb ($3.32/kg) (Ackerman, 2009).

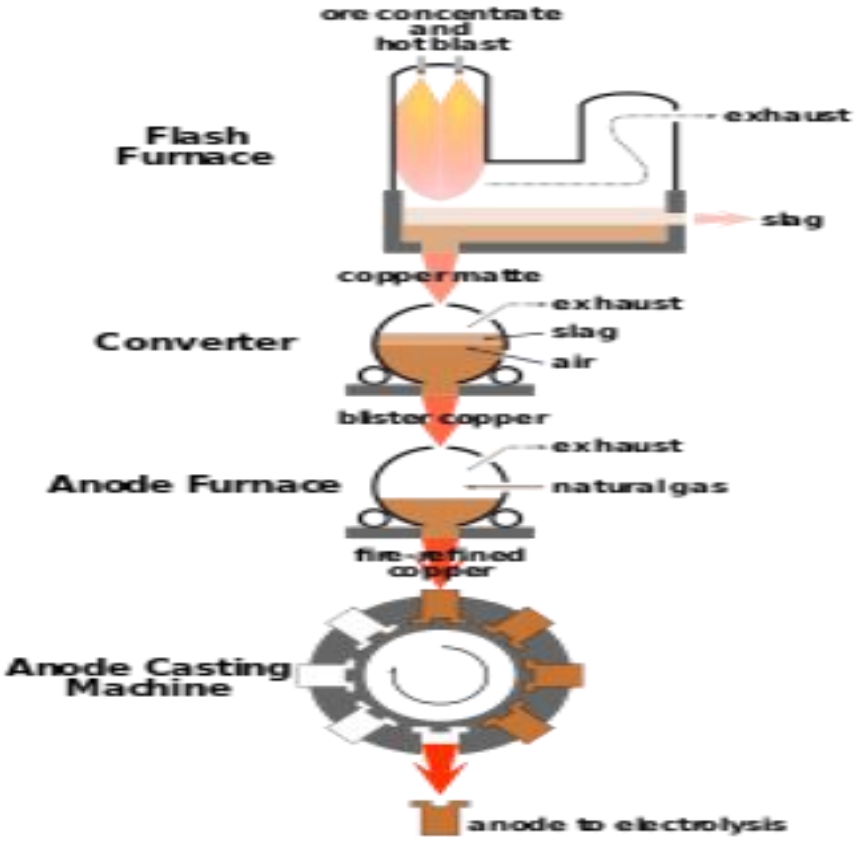


Figure 2.4: Scheme of flash smelting process

2.3.6 Method of copper extraction techniques

The concentration of copper in ores averages only 0.6%, and a large amount are minned commercial as ores of sulfides, especially chalcopyrite ($CuFeS_2$) and to a lesser extent chalcocite (Cu_2S) (Greenwood and Earnshaw 1997*)*. These minerals are concentrated from crushed ores to the level of 10–15% copper by froth flotation or bioleaching (Watling*,* 2006*)*. Heating this material with silica in flash smelting removes much of the iron as slag. The process exploits the greater ease of converting iron sulfides into oxides, which in turn react with the silica to form the silicate slag that floats on top of the heated mass. The resulting *copper matte,* consisting of Cu_2S, is roasted to convert all sulfides into oxides (Greenwood and Earnshaw 1997).

$$2\ Cu_2S + 3\ O_2 \rightarrow 2\ Cu_2O + 2\ SO_2$$

The cuprous oxide is converted to *blister* copper upon heating:

$$2\ Cu_2O \rightarrow 4\ Cu + O_2$$

The Sudbury matte process converted only half the sulfide to oxide and then used this oxide to remove the rest of the sulfur as oxide. It was then electrolytically refined and the anode mud exploited for the platinum and gold it contained. This step exploits the relatively easy reduction of copper oxides to copper metal. Natural gas is blown across the blister to remove most of the remaining oxygen and electrorefining is performed on the resulting material to produce pure copper (Samans*,* 1949)

$$Cu^{2+} + 2\ e^- \rightarrow Cu$$

2.3.8 Compounds of Copper

Copper forms a rich variety of compounds, usually with oxidation states +1 and +2, which are often called *cuprous* and *cupric*, respectively (Holleman and Wiberg, 2001).

2.3.9 Binary Compounds of Copper

As with other elements, the simplest compounds of copper are binary compounds, i.e. those containing only two elements, the principal examples being oxides, sulfides, and halides. Both cuprous and cupric oxides are known. Among the numerous copper sulfides, important examples include copper(I) sulfide and copper(II) sulfide.

Cuprous halides (with chlorine, bromine, and iodine) are known, as are cupric halides with fluorine, chlorine, and bromine. Attempts to prepare copper(II) iodide yield only cuprous iodide and iodine (Holleman and Wiberg 2001).

$$2\ Cu^{2+} + 4\ I^- \rightarrow 2\ CuI + I_2$$

2.3.10 Coordination chemistry of copper

Copper(II) gives a deep blue coloration in the presence of ammonia ligands. The one used here is tetramminecopper(II) sulfate. Copper forms coordination complexes with ligands. In aqueous solution, copper(II) exists as $[Cu(H_2O)_6]^{2+}$. This complex exhibits the fastest water exchange rate (speed of water ligands attaching and detaching) for any transition metal aquo complex. Adding aqueous sodium hydroxide causes the precipitation of light blue solid copper(II) hydroxide. A simplified equation is:

$$Cu^{2+} + 2\ OH^- \rightarrow Cu(OH)_2$$

Aqueous ammonia results in the same precipitate. Upon adding excess ammonia, the precipitate dissolves, forming tetraamminecopper(II)

$$Cu(H_2O)_4(OH)_2 + 4\ NH_3 \rightarrow [Cu(H_2O)_2(NH_3)_4]^{2+} + 2\ H_2O + 2\ OH^-$$

Many other oxyanions form complexes; these include copper(II) acetate, copper(II) nitrate, and copper(II) carbonate. Copper(II) sulfate forms a blue crystalline pentahydrate, the most familiar

copper compound in the laboratory. It is used in a fungicide called the Bordeaux mixture (Wiley-Vch, 2007).

Polyols, compounds containing more than one alcohol functional group, generally interact with cupric salts. For example, copper salts are used to test for reducing sugars. Specifically, using Benedict's reagent and Fehling's solution the presence of the sugar is signaled by a color change from blue Cu(II) to reddish copper(I) oxide (Ralph *et al*., 1999). Schweizer's reagent and related complexes with ethylenediamine and other amines dissolve cellulose (Saalwächter *et al.,* 2000). Amino acids form very stable chelate complexes with copper(II). Many wet-chemical tests for copper ions exist, one involving potassium ferrocyanide, which gives a brown precipitate with copper(II) salts.

2.3.11 Organocopper Chemistry of Copper

Compounds that contain a carbon-copper bond are known as organocopper compounds. They are very reactive towards oxygen to form copper(I) oxide and have many uses in chemistry. They are synthesized by treating copper(I) compounds with Grignard reagents, terminal alkynes or organolithium reagents (Norbert Krause, 2002) in particular, the last reaction described produces a Gilman reagent. These can undergo substitution with alkyl halides to form coupling products; as such, they are important in the field of organic synthesis. Copper(I) acetylide is highly shock-sensitive but is an intermediate in reactions such as the Cadiot-Chodkiewicz coupling (Berná *et al.,* 2008) and the Sonogashira coupling (Rafael and Carmen, 2007). Conjugate addition to enones and carbocupration of alkynes *(*Kharasch and tawney 1941) can also be achieved with organocopper compounds. Copper(I) forms a variety of weak complexes with alkenes and carbon monoxide, especially in the presence of amine ligands (Imai *et al.,* 1998).

2.3.12 Copper(III) and Copper(IV)

Copper(III) is most often found in oxides. A simple example is potassium cuprate, $KCuO_2$, a blue-black solid (Brauer, 1963). The most extensively studied copper(III) compounds are the cuprate superconductors. Yttrium barium copper oxide ($YBa_2Cu_3O_7$) consists of both Cu(II) and Cu(III) centres. Like oxide, fluoride is a highly basic anion (Schwesinger *et al.,* 2006) and is known to stabilize metal ions in high oxidation states. Both copper(III) and even copper(IV) fluorides are known, K_3CuF_6 and Cs_2CuF_6, respectively (Holleman and Wiberg, 2001).

Some copper proteins form oxo complexes, which also feature copper(III) (Lewis and Tolmain, 2004). With tetrapeptides, purple-colored copper(III) complexes are stabilized by the deprotonated amide ligands (McDonald *et al.,* 1997).

Complexes of copper(III) are also found as intermediates in reactions of organocopper compounds (Greenwood and Earnshaw 1997). For example, in the Kharasch–Sosnovsky reaction

2.3.13 Modern Period

Acid mine drainage affecting the stream running from the disused Parys Mountain copper mines The Great Copper Mountain was a mine in Falun, Sweden, that operated from the 10th century to 1992. It satisfied two thirds of Europe's copper consumption in the 17th century and helped fund many of Sweden's wars during that time (Lynch, 2004).

Copper is used in roofing (Grieken and Janssens, 2005), currency, and for photographic technology known as the daguerreotype. Copper was used in Renaissance sculpture, and was used to construct the Statue of Liberty; copper continues to be used in construction of various types. The Norddeutsche Affinerie in Hamburg was the first modern electroplating plant, starting its production in 1876 (Stelter and Bombach, 2004). The German scientist Gottfried Osann

invented powder metallurgy in 1830 while determining the metal's atomic mass; around then it was discovered that the amount and type of alloying element (e.g., tin) to copper would affect bell tones.

The Intergovernmental Council of Copper Exporting Countries, formed in 1967 by Chile, Peru, Zaire and Zambia, operated in the copper market as OPEC does in oil, though it never achieved the same influence, particularly because the second-largest producer, the United States, was never a member; it was dissolved in 1988 (Karen, 1976).

2.3.14 Folk Medicine of Copper

Copper is commonly used in jewelry, and according to some folklore, copper bracelets relieve arthritis symptoms (Richmond *et al.,* 2013). In one trial for osteoarthritis and one trial for rheumatoid arthritis no differences is found between copper bracelet and control (non-copper) bracelet (Richmond *et al.,* 2009).

2.3.15 Other Uses of Copper

Solutions of copper compounds are used as a wood preservative, particularly in treating the original portion of structures during restoration of dry rot damage. Together with zinc, copper wires may be installed over non-conductive roofing materials to discourage the growth of moss (Geoffrey*,* 2010). Textile fibers are blended with copper to create antimicrobial protective fabrics (Geoffrey*,* 2010). Copper alloys are used in musical instruments, particularly: the body of brass instruments; circuitry for all those that are electronically amplified; the bodies of brass percussion such as gongs, bells, and kettle drums; tuning heads on guitars and other string instruments; string windings on harps, pianos, harpsichords, and string instruments; and the

frame elements of pianos and harps. Copper is commonly used as a base on which other metals such as nickel are electroplated.

Copper is one of three metals, along with lead and silver, used in the museum materials testing procedure called the Oddy test to detect chlorides, oxides, and sulfur compounds.

Copper is used as the printing plate in etching, engraving and other forms of intaglio printmaking.

Copper oxide and carbonate are used add color in stained glass works, in glassmaking, and in ceramic glazes to impart turquoise blue, green, and brown colors.

Copper is used to create stills for distilling spirits, for example to make whisky. Its malleability makes it easy to bend into the various shapes required and allows considerable flexibility in the shaping of the still and associated pipework; the metal also reacts with undesirable sulfur-containing components in the vapor and distillate making for a cleaner product.

2.3.16 Degradation of Copper

Chromobacterium violaceum and *Pseudomonas fluorescens* can both mobilize solid copper as a cyanide compound (Harbhajan, 2006). The ericoid mycorrhizal fungi associated with *Calluna*, *Erica* and *Vaccinium* can grow in metalliferous soils containing copper (Harbhajan, 2006). The ectomycorrhizal fungus *Suillus luteus* protects young pine trees from copper toxicity. A sample of the fungus *Aspergillus niger* was found growing from gold mining solution and was found to contain cyano complexes of such metals as gold, silver, copper, iron, and zinc. The fungus also plays a role in the solubilization of heavy metal sulfides (Vest *et al.,* 2013).

2.3.17 Biological Role of Copper

Rich sources of copper include oysters, beef and lamb liver, Brazil nuts, blackstrap molasses, cocoa, and black pepper. Good sources include lobster, nuts and sunflower seeds, green olives, avocados, and wheat bran.

Copper proteins have diverse roles in biological electron transport and oxygen transportation, processes that exploit the easy interconversion of Cu(I) and Cu(II). Copper is essential in the aerobic respiration of all eukaryotes. In mitochondria, it is found in cytochrome c oxidase, which is the last protein in oxidative phosphorylation. Cytochrome c oxidase is the protein that binds the O_2 between a copper and an iron; the protein transfers 8 electrons to the O_2 molecule to reduce it to two molecules of water. Copper is also found in many superoxide dismutases, proteins that catalyze the decomposition of superoxides by converting it (by disproportionation) to oxygen and hydrogen peroxide:

$$2\ HO_2 \rightarrow H_2O_2 + O_2$$

The protein hemocyanin is the oxygen carrier in most mollusks and some arthropods such as the horseshoe crab (*Limulus polyphemus*) (Lippard and Berg, 1994). Because hemocyanin is blue, these organisms have blue blood rather than the red blood of iron-based hemoglobin. Structurally related to hemocyanin are the laccases and tyrosinases. Instead of reversibly binding oxygen, these proteins hydroxylate substrates, illustrated by their role in the formation of lacquers (Decker and Terwilliger, 2000). The biological role for copper commenced with the appearance of oxygen in earth's atmosphere (Schneider *et al.,* 2014). Several copper proteins, such as the "blue copper proteins", do not interact directly with substrates, hence they are not enzymes. These proteins relay electrons by the process called electron transfer (Decker and Terwilliger, 2000).

2.3.18 Dietary Needs of Copper

Copper is an essential trace element in plants and animals, but not in all microorganisms. The human body contains copper at a level of about 1.4 to 2.1 mg per kg of body mass (Linder *et al.,* 1998*)*. Copper is absorbed in the gut, then transported to the liver bound to albumin (Frieden and Hsieh 1976*).* After processing in the liver, copper is distributed to other tissues in a second phase, which involves the protein ceruloplasmin, carrying the majority of copper in blood. Ceruloplasmin also carries the copper that is excreted in milk, and is particularly well-absorbed as a copper source (Percival and Harris 1990). Copper in the body normally undergoes enterohepatic circulation (about 5 mg a day, vs. about 1 mg per day absorbed in the diet and excreted from the body), and the body is able to excrete some excess copper.

For U.S. food and dietary supplement labeling purposes the amount in a serving is expressed as a percent of Daily Value (%DV). For copper labeling purposes 100% of the Daily Value was 2.0 mg, but as of May 27, 2016 it was revised to 0.9 mg to bring it into agreement with the RDA (Bonham *et al.,* 2002*)*. A table of the old and new adult Daily Values is provided at Reference Daily Intake. The original deadline to be in compliance was July 28, 2018, but on September 29, 2017 the FDA released a proposed rule that extended the deadline to January 1, 2020 for large companies and January 1, 2021 for small companies (Li, 1994).

2.3.19 Copper Deficiency in Human

Because of its role in facilitating iron uptake, copper deficiency can produce anemia-like symptoms, neutropenia, bone abnormalities, hypopigmentation, impaired growth, increased incidence of infections, osteoporosis, hyperthyroidism, and abnormalities in glucose and cholesterol metabolism. Conversely, Wilson's disease causes an accumulation of copper in body tissues.

Severe deficiency can be found by testing for low plasma or serum copper levels, low ceruloplasmin, and low red blood cell superoxide dismutase levels; these are not sensitive to marginal copper status. The "cytochrome c oxidase activity of leucocytes and platelets" has been stated as another factor in deficiency, but the results have not been confirmed by replication (Gordon and John, 1986).

2.3.20 Copper Toxicity

Gram quantities of various copper salts have been taken in suicide attempts and produced acute copper toxicity in humans, possibly due to redox cycling and the generation of reactive oxygen species that damage DNA (Hunt and William, 1965). Corresponding amounts of copper salts (30 mg/kg) are toxic in animals (Ayyat *et al.,* 1995). A minimum dietary value for healthy growth in rabbits has been reported to be at least 3 ppm in the diet (Brewer, 2012).

Chronic copper toxicity does not normally occur in humans because of transport systems that regulate absorption and excretion. Autosomal recessive mutations in copper transport proteins can disable these systems, leading to Wilson's disease with copper accumulation and cirrhosis of the liver in persons who have inherited two defective genes (Linder *et al.,* 1998).

2.4 Selenium

Selenium is a chemical element with symbol Se and atomic number 34. It is a nonmetal with properties that are intermediate between the elements above and below in the periodic table, sulfur and tellurium, and also has similarities to arsenic. It rarely occurs in its elemental state or as pure ore compounds in the Earth's crust. Selenium (Greek σελήνη *selene* meaning "Moon")

was discovered in 1817 by Jöns Jacob Berzelius, who noted the similarity of the new element to the previously discovered tellurium (named for the Earth).

Selenium is found in metal sulfide ores, where it partially replaces the sulfur. Commercially, selenium is produced as a byproduct in the refining of these ores, most often during production. Minerals that are pure selenide or selenate compounds are known but rare. The chief commercial uses for selenium today are glassmaking and pigments. Selenium is a semiconductor and is used in photocells. Applications in electronics, once important, have been mostly replaced with silicon semiconductor devices. Selenium is still used in a few types of DC power surge protectors and one type of fluorescent quantum dot.

Selenium salts are toxic in large amounts, but trace amounts are necessary for cellular function in many organisms, including all animals. Selenium is an ingredient in many multivitamins and other dietary supplements, including infant formula. It is a component of the antioxidant enzymes glutathione peroxidase and thioredoxin reductase (which indirectly reduce certain oxidized molecules in animals and some plants). It is also found in three deiodinase enzymes, which convert one thyroid hormone to another. Selenium requirements in plants differ by species, with some plants requiring relatively large amounts and others apparently requiring none (Ruyle, 2009).

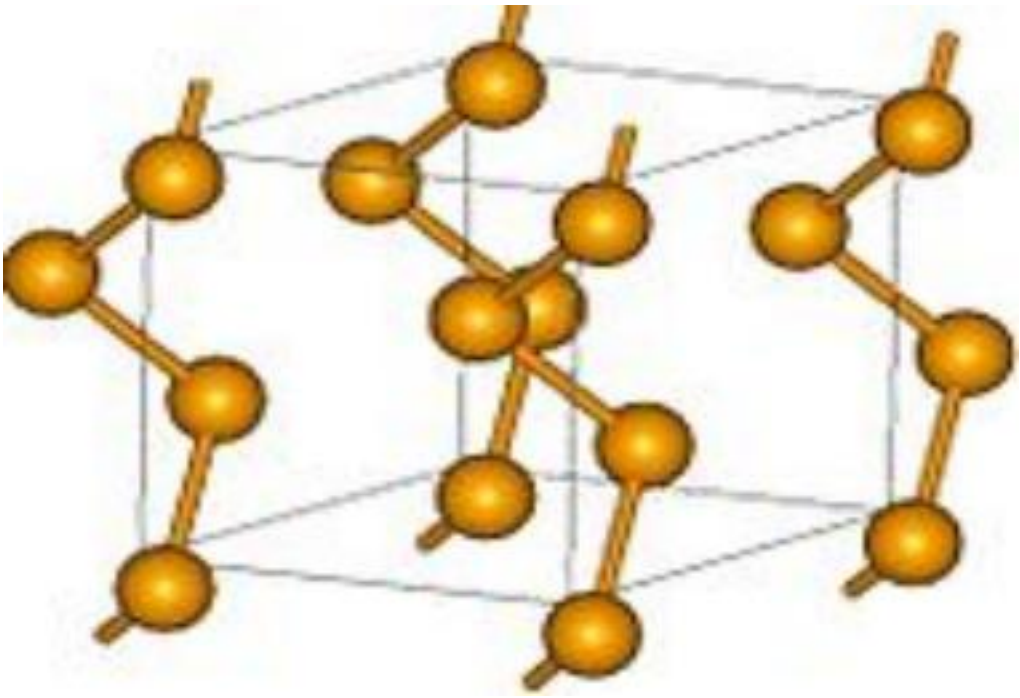


Figure 2.5: Structure of hexagonal (gray) selenium

2.4.1 Physical Properties of Selenium

Selenium forms several allotropes that interconvert with temperature changes, depending somewhat on the rate of temperature change. When prepared in chemical reactions, selenium is usually an amorphous, brick-red powder. When rapidly melted, it forms the black, vitreous form, usually sold commercially as beads (House, 2008). The structure of black selenium is irregular and complex and consists of polymeric rings with up to 1000 atoms per ring. Black Se is a brittle, lustrous solid that is slightly soluble in CS_2. Upon heating, it softens at 50 °C and converts to gray selenium at 180 °C; the transformation temperature is reduced by presence of halogens and amines (Greenwood and Earnshaw, 1997).

The red α, β, and γ forms are produced from solutions of black selenium by varying the evaporation rate of the solvent (usually CS_2). They all have relatively low, monoclinic crystal symmetries and contain nearly identical puckered Se_8 rings with different arrangements, as in sulfur. The packing is most dense in the α form. In the Se_8 rings, the Se-Se distance is 233.5 pm and Se-Se-Se angle is 105.7°. Other selenium allotropes may contain Se_6 or Se_7 rings (Greenwood and Earnshaw 1997).

The most stable and dense form of selenium is gray and has a hexagonal crystal lattice consisting of helical polymeric chains, where the Se-Se distance is 237.3 pm and Se-Se-Se angle is 130.1°. The minimum distance between chains is 343.6 pm. Gray Se is formed by mild heating of other allotropes, by slow cooling of molten Se, or by condensing Se vapor just below the melting point. Whereas other Se forms are insulators, gray Se is a semiconductor showing appreciable photoconductivity. Unlike the other allotropes, it is insoluble in CS_2 (Greenwood and Earnshaw 1997). It resists oxidation by air and is not attacked by nonoxidizing acids. With strong reducing

agents, it forms polyselenides. Selenium does not exhibit the changes in viscosity that sulfur undergoes when gradually heated (House, 2008).

2.4.2 Optical Properties of Selenium

Owing to its use as a photoconductor in flat-panel x-ray detectors, the optical properties of amorphous selenium (α-Se) thin films have been the subject of intense research (Jafar *et al.,* 2016*;* Saleh *et al.,* 2017*;* Minkov *et al.,* 2017).

2.4.3 Isotopes of Selenium

Selenium has seven natural isotopes, including ^{79}Se, which occurs in minute quantities in uranium ores, as well as 23 other synthetic isotopes.

Selenium isotopes of greatest stability

Isotope	Nature	Origin	Half-life
^{74}Se	Natural		Stable
^{76}Se	Natural		Stable
^{77}Se	Natural	Fission product	Stable
^{78}Se	Natural	Fission product	Stable
^{79}Se	Trace	Fission product	327000 yr (Jörg *et al.,* 2010*).*
^{80}Se	Natural	Fission product	Stable
^{82}Se	Natural	Fission product	~10^{20} yr (Audi *et al.,* 2003*)*

2.4.4 Chemical Compounds of Selenium

Selenium compounds commonly exist in the oxidation states −2, +2, +4, and +6.

2.4.5 Chalcogen Compounds

Selenium forms two oxides: selenium dioxide (SeO_2) and selenium trioxide (SeO_3). Selenium dioxide is formed by the reaction of elemental selenium with oxygen (House, 2008).

$$Se_8 + 8\ O_2 \rightarrow 8\ SeO_2$$

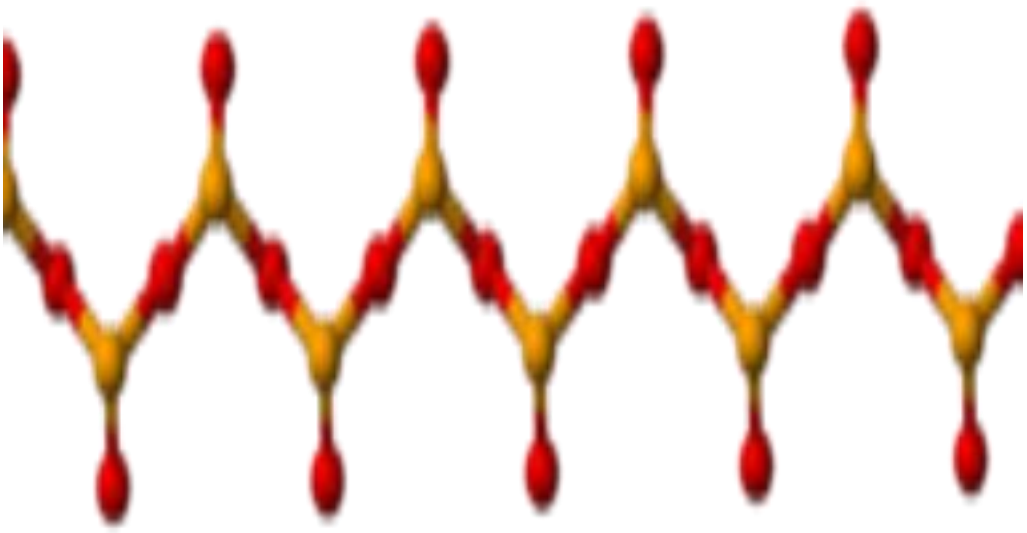


Figure 2.6: Structure of the polymer SeO_2

It is a polymeric solid that forms monomeric SeO_2 molecules in the gas phase. It dissolves in water to form selenous acid, H_2SeO_3. Selenous acid can also be made directly by oxidizing elemental selenium with nitric acid (Wiiberg *et al.,* 2001).

$$3\ Se + 4\ HNO_3 + H_2O \rightarrow 3\ H_2SeO_3 + 4\ NO$$

Unlike sulfur, which forms a stable trioxide, selenium trioxide is thermodynamically unstable and decomposes to the dioxide above 185 °C (House*,* 2008; Wiberg *et al.,* 2001).

$$2\ SeO_3 \rightarrow 2\ SeO_2 + O_2\ (\Delta H = -54\ kJ/mol)$$

Selenium trioxide is produced in the laboratory by the reaction of anhydrous potassium selenate (K_2SeO_4) and sulfur trioxide (SO_3) (Greenwood and Earnshaw, 1997).

Salts of selenous acid are called selenites. These include silver selenite (Ag_2SeO_3) and sodium selenite (Na_2SeO_3).

Hydrogen sulfide reacts with aqueous selenous acid to produce selenium disulfide:

$$H_2SeO_3 + 2\ H_2S \rightarrow SeS_2 + 3\ H_2O$$

Selenium disulfide consists of 8-membered rings. It has an approximate composition of SeS_2, with individual rings varying in composition, such as Se_4S_4 and Se_2S_6. Selenium disulfide has been used in shampoo as an antidandruff agent, an inhibitor in polymer chemistry, a glass dye, and a reducing agent in fireworks (Wiberg *et al.,* 2001).

Selenium trioxide may be synthesized by dehydrating selenic acid, H_2SeO_4, which is itself produced by the oxidation of selenium dioxide with hydrogen peroxide (Seppelt and Desmarteau, 1980).

$$SeO_2 + H_2O_2 \rightarrow H_2SeO_4$$

Hot, concentrated selenic acid can react with gold to form gold(III) selenate (Lenher*,* 1902)

2.4.6 Halogen Compounds of Selenium

Iodides of selenium are not well known. The only stable chloride is selenium monochloride (Se_2Cl_2), which might be better known as selenium(I) chloride; the corresponding bromide is also known. These species are structurally analogous to the corresponding disulfur dichloride. Selenium dichloride is an important reagent in the preparation of selenium compounds (e.g. the preparation of Se_7). It is prepared by treating selenium with sulfuryl chloride (SO_2Cl_2) (Xu, 2007). Selenium reacts with fluorine to form selenium hexafluoride:

$$Se_8 + 24\ F_2 \rightarrow 8\ SeF_6$$

In comparison with its sulfur counterpart (sulfur hexafluoride), selenium hexafluoride (SeF_6) is more reactive and is a toxic pulmonary irritant (Proctor and Hathaway 2004). Some of the selenium oxyhalides, such as selenium oxyfluoride ($SeOF_2$) and selenium oxychloride ($SeOCl_2$) have been used as specialty solvents (House, 2008).

2.4.7 Occurrence of Selenium

Selenium occurs naturally in a number of inorganic forms, including selenide, selenate, and selenite, but these minerals are rare. The common mineral selenite is not a selenium mineral, and contains no selenite ion, but is rather a type of gypsum (calcium sulfate hydrate) named like selenium for the moon well before the discovery of selenium. Selenium is most commonly found as an impurity, replacing a small part of the sulfur in sulfide ores of many metals (Kabata-Pendias, 1998; Fordyce, 2007).

In living systems, selenium is found in the amino acids selenomethionine, selenocysteine, and methylselenocysteine. In these compounds, selenium plays a role analogous to that of sulfur.

Another naturally occurring organoselenium compound is dimethyl selenide (Wessjohann *et al.,* 2007*;* Birringer *et al.,* 2002).

Certain solids are selenium-rich, and selenium can be bioconcentrated by some plants. In soils, selenium most often occurs in soluble forms such as selenate (analogous to sulfate), which are leached into rivers very easily by runoff (Kabata-Pendias*,* 1998*;* Fordyce*,* 2007). Ocean water contains significant amounts of selenium (Amouroux *et al.,* 2001*;* Haug *et al.,* 2007).

2.4.8 Production of Selenium

Industrial production of selenium usually involves the extraction of selenium dioxide from residues obtained during the purification of copper. Common production from the residue then begins by oxidation with sodium carbonate to produce selenium dioxide, which is mixed with water and acidified to form selenous acid (oxidation step). Selenous acid is bubbled with sulfur dioxide (reduction step) to give elemental selenium (Hoffmann*,* 1989; Hyvärinen *et al.,* 1989)

2.4.9 Applications of Selenium

2.4.9.1 Manganese Electrolysis of Selenium

During the electro winning of manganese, the addition of selenium dioxide decreases the power necessary to operate the electrolysis cells. China is the largest consumer of selenium dioxide for this purpose. For every tonne of manganese, an average 2 kg selenium oxide is used (Sun *et al.,* 2011).

2.4.9.2 Glass production

The largest commercial use of Se, accounting for about 50% of consumption, is for the production of glass. Se compounds confer a red color to glass. This color cancels out the green or yellow tints that arise from iron impurities typical for most glass. For this purpose, various selenite and selenate salts are added. For other applications, a red color may be desired, produced by mixtures of CdSe and CdS (Bernd *et al.,* 2005).

2.4.9.3 Alloys of Selenium

Selenium is used with bismuth in brasses to replace more toxic lead. The regulation of lead in drinking water applications with the Safe Drinking Water Act of 1974 made a reduction of lead in brass necessary. The new brass is marketed under the name EnviroBrass (Davis, 2001). Like lead and sulfur, selenium improves the machinability of steel at concentrations around 0.15% (Isakov, 2008; Gol'Dshtein *et al.,* 1979). Selenium produces the same machinability improvement in copper alloys (Davis, 2001).

2.4.9.4 Lithium–selenium batteries

Lithium–selenium (Li–Se) battery is one of the most promising system for energy storage in the family of lithium batteries (Eftekhari, 2017). Li–Se battery is an alternative to Lithium–sulfur battery with an advantage of high electrical conductivity.

2.4.9.5 Selenium in Solar Cells

Copper indium gallium selenide is a material used in solar cells (Deutsche, 2008). Amorphous selenium (α-Se) thin films have found application as photoconductors in flat panel x-ray detectors (Wee, 2006). These detectors utilize the amorphous selenium to capture and convert incident x-ray photons directly into electric charge (Wee, 2006). Based on this application, significant research has been undertaken in recent years to quantify the optical properties of such thin films (Jafar *et al.,* 2016*;* Saleh *et al.,* 2017*;* Minkov *et al.,* 2017*;* Springett, 1988).

2.4.10 Other Uses of selenium

Small amounts of organoselenium compounds are used to modify the vulcanization catalysts for the production of rubber (Naumov, 2010).

The photovoltaic and photoconductive properties are still valuable in photocopying (Springett, 1988*;* Williams, 2006*;* Diels and Arissian 2011*;* Meller and Grasser, 2009). Photocells, light meters and solar cells. Its use as a photoconductor in plain-paper copiers once was a leading application, but in the 1980s, the photoconductor application declined (although it was still a large end-use) as more and more copiers switched to organic photoconductors. Though once widely used, selenium rectifiers have mostly been replaced (or are being replaced) by silicon-based devices. The most notable exception is in power DC surge protection, where the superior energy capabilities of selenium suppressors make them more desirable than metal oxide varistors. Zinc selenide was the first material for blue LEDs, but gallium nitride is dominating the market now (Normile, 2000) Cadmium selenide was an important component in quantum dots. Sheets of amorphous selenium convert X-ray images to patterns of charge in xeroradiography and in solid-

state, flat-panel X-ray cameras (Kasap *et al.,* 2009). Ionized selenium (Se+24) is one of the active mediums used in X-ray lasers (Svelto, 1998).

Selenium is a catalyst in some chemical reactions, but it is not widely used because of issues with toxicity. In X-ray crystallography, incorporation of one or more selenium atoms in place of sulfur helps with multiple-wavelength anomalous dispersion and single wavelength anomalous dispersion phasing (Hai-fu *et al.,* 1993).

Selenium is used in the toning of photographic prints, and it is sold as a toner by numerous photographic manufacturers. Selenium intensifies and extends the tonal range of black-and-white photographic images and improves the permanence of prints (MacLean, 1937; Penichon, 1999; Mckenzie, 2003).

2.4.10.1 Biological Roles of Selenium

Although it is toxic in large doses, selenium is an essential micronutrient for animals. In plants, it occurs as a bystander mineral, sometimes in toxic proportions in forage (some plants may accumulate selenium as a defense against being eaten by animals, but other plants, such as locoweed, require selenium, and their growth indicates the presence of selenium in soil) (Ruyle, 2009).

The glutathione peroxidase family of enzymes (GSH-Px) catalyze certain reactions that remove reactive oxygen species such as hydrogen peroxide and organic hydroperoxides:

$$2\ GSH + H_2O_2 \text{----GSH-Px} \rightarrow GSSG + 2\ H_2O$$

The thyroid gland and every cell that uses thyroid hormone use selenium, which is a cofactor for the three of the four known types of thyroid hormone deiodinases, which activate and then deactivate various thyroid hormones and their metabolites; the iodothyronine deiodinases are the

subfamily of deiodinase enzymes that use selenium as the otherwise rare amino acid selenocysteine. (Only the deiodinase, iodotyrosine deiodinase, which works on the last breakdown products of thyroid hormone, does not use selenium (LPI, 2009).

Selenium may inhibit Hashimoto's disease, in which the body's own thyroid cells are attacked as alien. A reduction of 21% on TPO antibodies is reported with the dietary intake of 0.2 mg of selenium (Mazokopakis *et al.,* 2007).

Increased dietary selenium reduces the effects of mercury toxicity (Ralston *et al.,* 2008; Usuki *et al*., 2011 Penglase *et al.,* 2014), although it is effective only at low to modest doses of mercury (Ohi *et al.,* 1975). Evidence suggests that the molecular mechanisms of mercury toxicity includes the irreversible inhibition of selenoenzymes that are required to prevent and reverse oxidative damage in brain and endocrine tissues (Carvalho *et al.,* 2008; Ralston and Raymond, 2010). An antioxidant, selenoneine, which is derived from selenium and has been found to be present in the blood of bluefin tuna, is the subject of scientific research regarding its possible roles in inflammatory and chronic diseases, methylmercury detoxification, and oxidative damages (Yamashita *et al.,* 2010; Michiaki *et al.,* 2012)

2.4.11 Evolution in Biology Dietary Antioxidants

From about three billion years ago, prokaryotic selenoprotein families drive the evolution of selenocysteine, an amino acid. Selenium is incorporated into several prokaryotic selenoprotein families in bacteria, archaea, and eukaryotes as selenocysteine (Gladyshev *et al.,* 1999), where selenoprotein peroxiredoxins protect bacterial and eukaryotic cells against oxidative damage. Selenoprotein families of GSH-Px and the deiodinases of eukaryotic cells seem to have a bacterial phylogenetic origin. The selenocysteine-containing form occurs in species as diverse as

green algae, diatoms, sea urchin, fish, and chicken. Selenium enzymes are involved in the small reducing molecules glutathione and thioredoxin. One family of selenium-bearing molecules (the glutathione peroxidases) destroys peroxide and repairs damaged peroxidized cell membranes, using glutathione. Another selenium-bearing enzyme in some plants and in animals (thioredoxin reductase) generates reduced thioredoxin, a dithiol that serves as an electron source for peroxidases and also the important reducing enzyme ribonucleotide reductase that makes DNA precursors from RNA precursors (Stadtman, 1996).

Trace elements involved in GSH-Px and superoxide dismutase enzymes activities, i.e. selenium, vanadium, magnesium, copper, and zinc, may have been lacking in some terrestrial mineral-deficient areas (Gladyshec *et al.,* 1999). Marine organisms retained and sometimes expanded their selenoproteomes, whereas the selenoproteomes of some terrestrial organisms were reduced or completely lost. These findings suggest that, with the exception of vertebrates, aquatic life supports selenium use, whereas terrestrial habitats lead to reduced use of this trace element (Lobanov *et al.,* 2007). Marine fishes and vertebrate thyroid glands have the highest concentration of selenium and iodine. From about 500 million years ago, freshwater and terrestrial plants slowly optimized the production of "new" endogenous antioxidants such as ascorbic acid (vitamin C), polyphenols (including flavonoids), tocopherols, etc. The deiodinase isoenzymes constitute another family of eukaryotic selenoproteins with identified enzyme function. Deiodinases are able to extract electrons from iodides, and iodides from iodothyronines. They are, thus, involved in thyroid-hormone regulation, participating in the protection of thyrocytes from damage by H_2O_2 produced for thyroid-hormone biosynthesis (Venturi and Venturi, 2007*)*. About 200 million years ago, new selenoproteins were developed as mammalian

GSH-Px enzymes (Castellano *et al.,* 2004*;* Kryukov and Gladyshev, 2004*;* Wilting *et al.,* 1997*;* Zhang *et al.,* 2005).

2.4.12 Nutritional Sources of Selenium

Dietary selenium comes from nuts, cereals and mushrooms. Brazil nuts are the richest dietary source (though this is soil-dependent, since the Brazil nut does not require high levels of the element for its own needs) *(*Barclay *et al.,* 1995).

The U.S. Recommended Dietary Allowance (RDA) for teenagers and adults is 55 µg/day. Selenium as a dietary supplement is available in many forms, including multi-vitamins/mineral supplements, which typically contain 55 or 70 µg/serving. Selenium-specific supplements typically contain either 100 or 200 µg/serving.

In June 2015 the U.S. Food and Drug Administration (FDA) published its final rule establishing the requirement of minimum and maximum levels of selenium in infant formula (FDA, 2015).

The selenium content in the human body is believed to be in the 13–20 milligram range (Schroeder *et al.,* 1970; Zane, 2008).

2.4.13 Selenium Detection in Biological Fluids

Selenium may be measured in blood, plasma, serum, or urine to monitor excessive environmental or occupational exposure, to confirm a diagnosis of poisoning in hospitalized victims, or investigate a suspected case of fatal overdose. Some analytical techniques are capable of distinguishing organic from inorganic forms of the element. Both organic and inorganic forms of selenium are largely converted to monosaccharide conjugates (selenosugars) in the body prior

elimination in the urine. Cancer patients receiving daily oral doses of selenothionine may achieve very high plasma and urine selenium concentrations (Baselt, 2008).

2.4.14 Toxicity of Selenium

Although selenium is an essential trace element, it is toxic if taken in excess. Exceeding the Tolerable Upper Intake Level of 400 micrograms per day can lead to selenosis (NIH, 2009). This 400 µg Tolerable Upper Intake Level is based primarily on a 1986 study of five Chinese patients who exhibited overt signs of selenosis and a follow up study on the same five people in 1992 (FNB, 2000). The 1992 study actually found the maximum safe dietary Se intake to be approximately 800 micrograms per day (15 micrograms per kilogram body weight), but suggested 400 micrograms per day to avoid creating an imbalance of nutrients in the diet and to accord with data from other countries (Yang and Zhou, 1994). In China, people who ingested corn grown in extremely selenium-rich stony coal (carbonaceous shale) have suffered from selenium toxicity. This coal was shown to have selenium content as high as 9.1%, the highest concentration in coal ever recorded (Yang and Xia, 1995).

Signs and symptoms of selenosis include a garlic odor on the breath, gastrointestinal disorders, hair loss, sloughing of nails, fatigue, irritability, and neurological damage. Extreme cases of selenosis can exhibit cirrhosis of the liver, pulmonary edema, or death (Wilber, 1980). Elemental selenium and most metallic selenides have relatively low toxicities because of low bioavailability. By contrast, selenates and selenites have an oxidant mode of action similar to that of arsenic trioxide and are very toxic. The chronic toxic dose of selenite for humans is about 2400 to 3000 micrograms of selenium per day (Wilber, 1980). Hydrogen selenide is an extremely toxic, corrosive gas (Olson, 1986). Selenium also occurs in organic compounds, such as dimethyl

selenide, selenomethionine, selenocysteine and methylselenocysteine, all of which have high bioavailability and are toxic in large doses.

Selenium poisoning of water systems may result whenever new agricultural runoff courses through normally dry, undeveloped lands. This process leaches natural soluble selenium compounds (such as selenates) into the water, which may then be concentrated in new "wetlands" as the water evaporates. Selenium pollution of waterways also occurs when selenium is leached from coal flue ash, mining and metal smelting, crude oil processing, and landfill (Lemly, 2004) The resultant high selenium levels in waterways were found to cause congenital disorders in oviparous species, including wetland birds (Ohlendorf, 2003) and fish (Lemly, 1997) Elevated dietary methylmercury levels can amplify the harm of selenium toxicity in oviparous species (Penglase *et al.,* 2014)

In fish and other wildlife, selenium is necessary for life, but toxic in high doses. For salmon, the optimal concentration of selenium is about 1 microgram selenium per gram of whole body weight. Much below that level, young salmon die from deficiency much above, they die from toxic excess (Hamilton *et al.,* 1990).

The Occupational Safety and Health Administration (OSHA) has set the legal limit (Permissible exposure limit) for selenium in the workplace at 0.2 mg/m^3 over an 8-hour workday. The National Institute for Occupational Safety and Health (NIOSH) has set a Recommended exposure limit (REL) of 0.2 mg/m^3 over an 8-hour workday. At levels of 1 mg/m^3, selenium is immediately dangerous to life and health (CDC, 2005)

2.4.15 Deficiency of Selenium

Selenium deficiency can occur in patients with severely compromised intestinal function, those undergoing total parenteral nutrition, and (Ravaglia *et al.,* 2000).

Selenium deficiency, defined by low (<60% of normal) selenoenzyme activity levels in brain and endocrine tissues, occurs only when a low selenium level is linked with an additional stress, such as high exposures to mercury (Ralston and Raymond, 2010) or increased oxidant stress from vitamin E deficiency (Mann *et al.,* 2002).

Selenium interacts with other nutrients, such as iodine and vitamin E. The effect of selenium deficiency on health remains uncertain, particularly in relation to Kashin-Beck disease (Moreno-Reyes *et al.,* 2003). Also, selenium interacts with other minerals, such as zinc and copper. High doses of Se supplements in pregnant animals might disturb the Zn:Cu ratio and lead to Zn reduction; in such treatment cases, Zn levels should be monitored. Further studies are needed to confirm these interactions (Kachuee *et al.,* 2013).

In the regions (e.g. various regions within North America) where low selenium soil levels lead to low concentrations in the plants, some animal species may be deficient unless selenium is supplemented with diet or injection (National Research Council, Subcommittee on Sheep Nutrition 1985). Ruminants grazing certain forages, e.g., some white clover varieties containing cyanogenic glycosides, may have higher selenium requirements ((National Research Council, Committee on Nutrient Requirements of Small Ruminants, 2007) presumably because cyanide is released from the aglycone by glucosidase activity in the rumen (Coop and Blakely, 1949) and glutathione peroxidases is deactivated by the cyanide acting on the glutathione moiety (Kraus*,* 1980). Neonate ruminants at risk of white muscle disease may be administered both selenium

and vitamin E by injection; some of the WMD myopathies respond only to selenium, some only to vitamin E, and some to either (Kahn, 2005).

2.4.16 Controversial Health Effects of Selenium

A number of correlative epidemiological studies have implicated selenium deficiency (measured by blood levels) in a number of serious or chronic diseases, such as cancer (Ip, 1998; Amaral *et al.,* 2010), diabetes (Ip, C. 1998), HIV/AIDS (Rayman*,* 2000), and tuberculosis. In addition, selenium supplementation has been found to be a chemopreventive for some types of cancer in some types of rodents. One study of 118 exocrine pancreatic cancer (EPC) patients and 399 hospital controls in eastern Spain found high selenium concentrations to be inversely associated with the risk of EPC (Amaral *et al.,* 2012). However, in randomized, blinded, controlled prospective trials in humans, selenium supplementation has not succeeded in reducing the incidence of any disease, nor has a meta-analysis of such selenium supplementation studies detected a decrease in overall mortality (Bjelakovic *et al.,* 2012).

2.5 Vitamin D

Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, and multiple other biological effects. In humans, the most important compounds in this group are vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol) (Holick*,* 2006). Cholecalciferol and ergocalciferol can be ingested from the diet and from supplements (Calvo *et al.,* 2005; Holick, 2006; Norman*,* 2008). Only a few foods contain vitamin D. The major natural source of the vitamin is synthesis of cholecalciferol in the skin from cholesterol through a chemical reaction that is dependent on sun

exposure (specifically UVB radiation). Dietary recommendations typically assume that all of a person's vitamin D is taken by mouth, as sun exposure in the population is variable and recommendations about the amount of sun exposure that is safe are uncertain in view of the skin cancer risk.

Vitamin D from the diet or skin synthesis is biologically inactive; enzymatic conversion (hydroxylation) in the liver and kidney is required for activation. As vitamin D can be synthesized in adequate amounts by most mammals exposed to sufficient sunlight, it is not an essential dietary factor, and so not technically a vitamin (Norman, 2008). Instead it could be considered as a hormone, with activation of the vitamin D pro-hormone resulting in the active form, calcitriol, which then produces effects via a nuclear receptor in multiple different locations (Norman, 2008). Cholecalciferol is converted in the liver to calcifediol (25-hydroxycholecalciferol); ergocalciferol is converted to 25-hydroxyergocalciferol. These two vitamin D metabolites (called 25-hydroxyvitamin D or 25(OH)D) are measured in serum to determine a person's vitamin D status (Hollis, 1996) Calcifediol is further hydroxylated by the kidneys to form calcitriol (also known as 1,25-dihydroxycholecalciferol), the biologically active form of vitamin D (Holick *et al.,* 1971). Calcitriol circulates as a hormone in the blood, having a major role regulating the concentration of calcium and phosphate, and promoting the healthy growth and remodeling of bone. Calcitriol also has other effects, including some on cell growth, neuromuscular and immune functions, and reduction of inflammation (NIH, 2016).

Vitamin D has a significant role in calcium homeostasis and metabolism. Its discovery was due to effort to find the dietary substance lacking in children with rickets (the childhood form of osteomalacia) (Wolf, 2004). Vitamin D supplements are given to treat or to prevent osteomalacia and rickets, but the evidence for other health effects of vitamin D supplementation in the general

population is inconsistent (Pittas *et al.,* 2010; Chung *et al.,* 2009). The effect of vitamin D supplementation on mortality is not clear, with one meta-analysis finding a small decrease in mortality in elderly people (Bjelakovic *et al.,* 2014), and another concluding no clear justification exists for recommending supplementation for preventing many diseases, and that further research of similar design is unneeded in these areas (Bolland *et al.,* 2014).

2.5.1 Vitamin D Deficiency

A diet deficient in vitamin D in conjunction with inadequate sun exposure causes osteomalacia (or rickets when it occurs in children), which is a softening of the bones. In the developed world, this is a rare disease (NHS, 2012). However, vitamin D deficiency has become a worldwide problem in the elderly and remains common in children and adults (Eriksen and Glerup 2002*;* Holick*,* 2007) Low blood calcifediol (25-hydroxy-vitamin D) can result from avoiding the sun (Schoenmakers *et al.,* 2008*)*.[19] Deficiency results in impaired bone mineralization and bone damage which leads to bone-softening diseases (Grant *et al.,* 2005*;* Brown *et al.,* 2013) including rickets and osteomalacia.

2.5.1.1 Rickets

Rickets, a childhood disease, is characterized by impeded growth and soft, weak, deformed long bones that bend and bow under their weight as children start to walk. This condition is characterized by bow legs (Brown *et al.,* 2013) which can be caused by calcium or phosphorus deficiency, as well as a lack of vitamin D; today, it is largely found in low-income countries in Africa, Asia, or the Middle East (Brown *et al.,* 2013) and in those with genetic disorders such as pseudovitamin D deficiency rickets (Zargar *et al.,* 2000).

Maternal vitamin D deficiency may cause overt bone disease from before birth and impairment of bone quality after birth (Paterson *et al.,* 2015; Elidrissy *et al.,* 2016). Nutritional rickets exists in countries with intense year-round sunlight such as Nigeria and can occur without vitamin D deficiency (Oramasionwu *et al.,* 2008).

Although rickets and osteomalacia are now rare in Britain, outbreaks have happened in some immigrant communities in which osteomalacia sufferers included women with seemingly adequate daylight outdoor exposure wearing Western clothing (Dunnigan *et al.,* 1997). Having darker skin and reduced exposure to sunshine did not produce rickets unless the diet deviated from a Western omnivore pattern characterized by high intakes of meat, fish, and eggs, and low intakes of high-extraction cereals (Robertson *et al.,* 1981; Clements*,* 1989; Pettifor, 2004). The dietary risk factors for rickets include abstaining from animal foods (Robertson *et al.,* 1997; Dunnigan *et al.,* 2005).

Vitamin D deficiency remains the main cause of rickets among young infants in most countries, because breast milk is low in vitamin D and social customs and climatic conditions can prevent adequate sun exposure. In sunny countries such as Nigeria, South Africa, and Bangladesh, where rickets occurs among older toddlers and children, it has been attributed to low dietary calcium intakes, which are characteristic of cereal-based diets with limited access to dairy products (Pettifor*,* 2004).

Almost two-thirds of 500 children had mild rickets in the late 1920s (Weick*,* 1967). An increase in the proportion of animal protein (Garrison and Somer 1997; Dunnigan *et al.,* 2005) in the 20th century American diet coupled with increased consumption of milk (Dupuis, 2002; Teegarden *et al.,* 1999), fortified with relatively small quantities of vitamin D coincided with a dramatic decline in the number of rickets cases (Holick, 2004). Also, in the United States and Canada,

vitamin D-fortified milk, infant vitamin supplements, and vitamin supplements have helped to eradicate the majority of cases of rickets for children with fat malabsorption conditions (Brown *et al.,* 2013).

2.5.1.2 Osteomalacia

Osteomalacia is a disease in adults that results from vitamin D deficiency. Characteristics of this disease are softening of the bones, leading to bending of the spine, bowing of the legs, proximal muscle weakness, bone fragility, and increased risk for fractures (Insel *et al.,* 2015). Osteomalacia reduces calcium absorption and increases calcium loss from bone, which increases the risk for bone fractures. Osteomalacia is usually present when 25-hydroxyvitamin D levels are less than about 10 ng/mL (Holick*,* 2006). Although the effects of osteomalacia are thought to contribute to chronic musculoskeletal pain (Holick*,* 2003), there is no persuasive evidence of lower vitamin D levels in chronic pain sufferers (Straube *et al.,* 2009) or that supplementation alleviates chronic nonspecific musculoskeletal pain (Gaikwad *et al.*, 2016).

2.5.1.3 Skin pigmentation

Dark-skinned people living in temperate climates have been shown to have low vitamin D levels but the significance of this is not certain (Lowe and Bhojani 2017*;* O'Connor and Register 2012) Dark-skinned people may be less efficient at making vitamin D because melanin in the skin hinders vitamin D synthesis (Khalid *et al.,* 2017)

2.5.2 Effects of Vitamin D Suppliment

The effects of vitamin D supplementation on health are uncertain (Chung *et al.,* 2009*;* Theodoratou *et al.,* 2014). A 2013 review did not find any effect from supplementation on the rates of disease, other than a tentative decrease in mortality in the elderly (Autier *et al.,* 2014). Low vitamin D levels may result from disease rather than cause disease (Autier *et al.,* 2014).

A United States Institute of Medicine report states: "Outcomes related to cancer, cardiovascular disease and hypertension, and diabetes and metabolic syndrome, falls and physical performance, immune functioning and autoimmune disorders, infections, neuropsychological functioning, and preeclampsia could not be linked reliably with calcium or vitamin D intake and were often conflicting (Ross *et al.,* 2011). Some researchers claim the IOM was too definitive in its recommendations and made a mathematical mistake when calculating the blood level of vitamin D associated with bone health (Maxmen*,* 2011). Members of the IOM panel maintain that they used a "standard procedure for dietary recommendations" and that the report is solidly based on the data. Research on vitamin D supplements, including large-scale clinical trials, is continuing (Maxmen*,* 2011).

Vitamin D supplements do not alter the outcomes for myocardial infarction, stroke or cerebrovascular disease, cancer, bone fractures or knee osteoarthritis (Bolland *et al.,* 2014*;* Hussain *et al.,* 2017).

2.5.3 Mortality

Vitamin D_3 supplementation has been tentatively found to lead to a reduced risk of death in the elderly (Bjelakovic *et al.,* 2014*;* Autier *et al.,* 2014) but the effect has not been deemed pronounced or certain enough to make taking supplements recommendable (Bolland *et al.,* 2014). Other forms (Vitamin D_2, alfacalcidol, and calcitriol) do not appear to have any beneficial effects with regard to the risk of death (Bjelakovic *et al.,* 2014). High blood levels appear to be associated with a lower risk of death, but it is unclear if supplementation can result in this benefit (Schöttker *et al.,* 2014) Both an excess and a deficiency in vitamin D appear to cause abnormal functioning and premature aging (Tuohimaa*,* 2009*;* Tuohimaa *et al.,* 2009*;* Manya *et al.,* 2010). The relationship between serum calcifediol level and all-cause mortality is parabolic (*Rioss et al.,* 2011). Harm from vitamin D appears to occur at a lower vitamin D level in the black population than in the white population (Ross *et al.,* 2011).

2.5.4 Maintenance of Bone in Health

In general, no good evidence supports the commonly held belief that vitamin D supplements can help prevent osteoporosis (Bolland *et al.,* 2014). Its general use for prevention of this disease in those without vitamin D deficiency is thus likely not needed (Reid *et al.,* 2014).

For older people with osteoporosis, taking vitamin D with calcium may help prevent hip fractures, but it also slightly increases the risk of stomach and kidney problems (Avenell *et al.,* 2014). Supplementation with higher doses of vitamin D, in those older than 65 years, may decrease fracture risk (Bischoff-Ferrari *et al.,* 2012). The effect may be smaller for people living independently than for people in institutions (Chung *et al.,* 2011).

Vitamin D deficiency causes osteomalacia (called rickets when it occurs in children). Use of vitamin D in children with normal vitamin D levels does not appear to improve bone density (Winzenberg *et al.,* 2011). Beyond that, low serum vitamin D levels have been associated with falls, and low bone mineral density (Cranney *et al.,* 2007). Taking extra vitamin D, however, does not appear to change the risk (Bolland *et al.,* 2014).

Athletes who are vitamin D deficient are at an increased risk of stress fractures and/or major breaks, particularly those engaging in contact sports. The greatest benefit with supplementation is seen in athletes who are deficient (25(OH)D serum levels <30 ng/ml), or severely deficient (25(OH)D serum levels <25 ng/ml). Incremental decreases in risks are observed with rising serum 25(OH)D concentrations plateauing at 50 ng/ml with no additional benefits seen in levels beyond this point (Shuler *et al.,* 2012).

2.5.5 Cancer and Vitamin D

Vitamin D supplements have been widely marketed for their claimed anticancer properties (Byers*,* 2010). Associations have been shown in observational studies between low vitamin D levels and the risk of development of certain cancers including colon cancer (Feldman *et al.,,* 2014).

It is unclear, however, if taking additional vitamin D in the diet or as supplements affects the risk of cancer. Reviews have described the evidence as being "inconsistent, inconclusive as to causality, and insufficient to inform nutritional requirements" (Ross *et al.,* 2011) and "not sufficiently robust to draw conclusions" (Chung *et al.,* 2011).

One 2014 review found that supplements had no significant effect on cancer risk (Bolland *et al.,* 2014). Another 2014 review concluded that vitamin D_3 may decrease the risk of death from

cancer (one fewer death in 150 people treated over 5 years), but concerns with the quality of the data were noted (Bjelakovic *et al.,* 2014).

Insufficient evidence exists to recommend vitamin D supplements for people with cancer, although some evidence suggests that low vitamin D may be associated with a worse outcome for some cancers (Buttigliero *et al.,* 2011) and that higher 25-hydroxy vitamin D levels at the time of diagnosis are associated with better outcomes (Li *et al.,* 2014).

2.5.6 Cardiovascular Disease

Taking vitamin D supplements does not meaningfully reduce the risk of stroke, cerebrovascular disease, cardial infarction, or ischaemic heart disease (Bolland *et al.,* 2014) Supplementation has no effect on blood pressure (Beveridge *et al.,* 2015)

2.5.7 Protection of Immune System against Infectious Diseases

In general, vitamin D functions to activate the innate and dampen the adaptive immune systems (Hewison*,* 2011). Deficiency has been linked to increased risk of viral infections, including HIV and influenza (Beard *et al.,* 2011; Spector*,* 2011; Cannell *et al.,* 2006). Low levels of vitamin D appear to be a risk factor for tuberculosis (Nnoiaham and Clarke 2008) and historically it was used as a treatment (Luong *et al.,* 2011).

Supplementation slightly decreases the risk of respiratory tract infections and the exacerbation of asthma (Bergman *et al.,* 2013; Martineau *et al.,* 2017; Autier *et al.,* 2017). Evidence is lacking on whether it does so in children under five years of age (Yakoob *et al.,* 2016). No clinical trials have been done to assess its effect on preventing other infections, such as malaria.

2.5.8 Autoimmune Disease

Although tentative data link low levels of vitamin D to asthma, evidence to support a beneficial effect on asthmatics from supplementation is inconclusive (Hart, 2012) Accordingly, supplementation is not currently recommended for treatment or prevention of asthma (Paul *et al.,* 2012).

Vitamin D and multiple sclerosis incidence have been linked, but it is not clear what the nature of any causal relationship might be (Pakpoor *et al.,* 2014). There is no evidence that vitamin D supplementation is helpful for treating people with multiple sclerosis (Pozuelo-Moyano *et al.,* 2013). Low levels of vitamin D are associated with Crohn's disease and ulcerative colitis (Del Pinto *et al.,* 2015). Further studies are required to determine its significance (Del Pinto *et al.,* 2015).

2.5.9 Other Health Condition

2.5.9.1 Diabetes

A systematic review of 2014 concluded that the available studies show no evidence of vitamin D3 supplementation having an effect on glucose homeostasis or diabetes prevention (Seida *et al.,* 2014). A review article of 2016 reported that while there is increasing evidence that vitamin D deficiency may be a risk factor for diabetes, over-all evidence regarding vitamin D levels and diabetes mellitus is contradictory, requiring further studies (Nakashima *et al.,* 2016).

2.5.9.2 Depression

Clinical trials of vitamin D supplementation for depressive symptoms have generally been of low quality and show no overall effect, although subgroup analysis showed supplementation for participants with clinically significant depressive symptoms or depressive disorder had a moderate effect (Shaffer *et al.,* 2014).

2.5.9.3 Cognition and Demential

A systematic review of clinical studies found an association between low vitamin D levels, and cognitive impairment and a higher risk of developing Alzheimer's disease. However, lower vitamin D concentrations are also associated with poor nutrition and spending less time outdoors. Therefore, alternative explanations for the increase in cognitive impairment exist and hence a direct causal relationship between vitamin D levels and cognition could not be established (Balion *et al.,* 2012).

2.5.9.4 Pregnancy

Low levels of vitamin D in pregnancy are associated with gestational diabetes, pre-eclampsia, and small infants (Aghajafari *et al.,* 2013). Although taking vitamin D supplements during pregnancy raises blood levels of vitamin D in the mother at term (*Palacios et al., 2016*) the extent of benefits for the mother or fetus is unclear (Aghajafari *et al.,* 2013*;* Palacios *et al.,* 2016*;* Roth *et al.,* 2017). Pregnant women who take an adequate amount of vitamin D during gestation may experience a lower risk of pre-eclampsia (Palacios *et al.,* 2016) and positive immune effects (Wagner *et al.,* 2012). Pregnant women often do not take the recommended amount of vitamin D (Wagner *et al.,* 2012).

2.5.9.5 Weight Loss

Though hypothesized that vitamin D supplementation may be an effective treatment for obesity apart from calorie restriction, one systematic review found no association of supplementation with body weight or fat mass (Pathak *et al.,* 2014). A 2016 meta-analysis found that circulating vitamin D status was improved by weight loss, indicating that fat mass may be inversely associated with blood levels of vitamin D (Pathak *et al.,* 2014).

2.5.10 Recommended Serum Level of Vitamin D

A 2014 review concluded that the most advantageous serum levels for 25(OH)D for all outcomes appeared to be close to 30 ng/ml (75 nmol/L) (Bischoff-Ferrari, 2014). The optimal vitamin D levels are still controversial and another review concluded that ranges from 30 to 40 ng/ml (75 to 100 nmol/L) were to be recommended for athletes (Dahlquist *et al.,* 2015). Part of the controversy is because numerous studies have found differences in serum levels of 25(OH)D between ethnic groups; studies point to genetic as well as environmental reasons behind these variations (Engelman *et al.,* 2008). Supplementation to achieve these standard levels could cause harmful vascular calcification (Freedman and Register, 2012).

In 2011 an IOM committee concluded a serum 25(OH)D level of 20 ng/ml (50 nmol/L) is needed for bone and overall health. The dietary reference intakes for vitamin D are chosen with a margin of safety and 'overshoot' the targeted serum value to ensure the specified levels of intake achieve the desired serum 25(OH)D levels in almost all persons. No contributions to serum 25(OH)D level are assumed from sun exposure and the recommendations are fully applicable to people with dark skin or negligible exposure to sunlight. The Institute found serum 25(OH)D concentrations above 30 ng/ml (75 nmol/L) are "not consistently associated with increased

benefit". Serum 25(OH)D levels above 50 ng/ml (125 nmol/L) may be cause for concern. However, some people with serum 25(OH)D between 30 and 50 ng/ml (75 nmol/L-125 nmol/L) will also have inadequate vitamin D (Ross *et al.,* 2011).

The risk of cardiovascular disease is lower when vitamin D ranged from 8 to 24 ng/ml (20 to 60 nmol/L). A "threshold effect" appears to occur once a level of 24 ng/ml (60 nmol/L) has been reached i.e., levels of vitamin D over 24 ng/ml (60 nmol/L) did not show added benefit (Wang *et al.,* 2012).

2.5.11 Excess of Vitamin D in the Body

Vitamin D toxicity is rare (Holick, 2007). It is caused by supplementing with high doses of vitamin D rather than sunlight. The threshold for vitamin D toxicity has not been established; however, according to some research, the tolerable upper intake level (UL) is 4,000 IU/day for ages 9–71 (Ross *et al.,* 2011) (100 µg/day), while other research concludes that, in healthy adults, sustained intake of more than 1250 µg/day (50,000 IU) can produce overt toxicity after several months and can increase serum 25-hydroxyvitamin D levels to 150 ng/ml and greater (Holick, 2007). Those with certain medical conditions, such as primary hyperparathyroidism (Vieth, 1999) are far more sensitive to vitamin D and develop hypercalcemia in response to any increase in vitamin D nutrition, while maternal hypercalcemia during pregnancy may increase fetal sensitivity to effects of vitamin D and lead to a syndrome of mental retardation and facial deformities (Vieth, 1999).

A review published in 2015 noted that adverse effects have been reported only at 25(OH)D serum concentrations above 200 nmol/L (Dahlquist *et al.,* 2015).

Published cases of toxicity involving hypercalcemia in which the vitamin D dose and the 25-hydroxy-vitamin D levels are known all involve an intake of ≥40,000 IU (1,000 μg) per day (Vieth, 1999).

Pregnant or breastfeeding women should consult a doctor before taking a vitamin D supplement. The FDA advised manufacturers of liquid vitamin D supplements that droppers accompanying these products should be clearly and accurately marked for 400 international units (1 IU is the biological equivalent of 25 ng cholecalciferol/ergocalciferol). In addition, for products intended for infants, the FDA recommends the dropper hold no more than 400 IU (DeLancey, 2010). After being commissioned by the Canadian and American governments, the Institute of Medicine (IOM) as of 30 November 2010, has increased the tolerable upper limit (UL) to 2,500 IU per day for ages 1–3 years, 3,000 IU per day for ages 4–8 years and 4,000 IU per day for ages 9–71+ years (including pregnant or lactating women) (Ross *et al.,* 2012). Calcitriol itself is auto-regulated in a negative feedback cycle, and is also affected by parathyroid hormone, fibroblast growth factor 23, cytokines, calcium, and phosphate (Olmos-Ortiz *et al.,* 2015).

2.5.12 Toxicity Effect of Vitamin D

Vitamin D overdose causes hypercalcemia, which is a strong indication of vitamin D toxicity – this can be noted with an increase in urination and thirst. If hypercalcemia is not treated, it results in excess deposits of calcium in soft tissues and organs such as the kidneys, liver, and heart, resulting in pain and organ damage (Holick, 2007; Brown *et al.,* 2013; Insel *et al.,* 2015).

The main symptoms of vitamin D overdose which are those of hypercalcemia including anorexia, nausea, and vomiting. These may be followed by polyuria, polydipsia, weakness, insomnia, nervousness, pruritus and ultimately renal failure. Furthermore, proteinuria, urinary casts,

azotemia, and metastatic calcification (especially in the kidneys) may develop. Other symptoms of vitamin D toxicity include mental retardation in young children, abnormal bone growth and formation, diarrhea, irritability, weight loss, and severe depression (Holick, 2007; Insel *et al.,* 2015).

Vitamin D toxicity is treated by discontinuing vitamin D supplementation and restricting calcium intake. Kidney damage may be irreversible. Exposure to sunlight for extended periods of time does not normally cause vitamin D toxicity. The concentrations of vitamin D precursors produced in the skin reach an equilibrium, and any further vitamin D produced is degraded (Vieth, 1999).

2.5.13 Biosynthesis of Vitamin D

Synthesis of vitamin D in nature is dependent on the presence of UV radiation and subsequent activation in liver and in kidney. Many animals synthesize vitamin D_3 from 7-dehydrocholesterol, and many fungi synthesize vitamin D_2 from ergosterol (Holick, 1992; Keegan *et al.,* 2013).

2.5.14 Interactive Pathway of Vitamin D

The transformation that converts 7-dehydrocholesterol to vitamin D_3 occurs in two steps (Holick, 1987: Deluca, 2014). First, 7-dehydrocholesterol is photolyzed by ultraviolet light in a 6-electron conrotatory ring-opening electrocyclic reaction; the product is previtamin D_3. Second, previtamin D_3 spontaneously isomerizes to vitamin D_3 (cholecalciferol) in an antarafacial sigmatropic [1,7] hydride shift. At room temperature, the transformation of previtamin D_3 to vitamin D_3 in an organic solvent takes about 12 days to complete. The conversion of

previtamin D_3 to vitamin D_3 in the skin is about 10 times faster than in an organic solvent (Holick, 2004).

2.5.15 Vitamin D Synthesis in Skin

In the epidermal strata of the skin, vitamin D production is greatest in the stratum basale (colored red in the illustration) and stratum spinosum (colored light brown).

Vitamin D_3 is produced photochemically from 7-dehydrocholesterol in the skin of most vertebrate animals, including humans (Crissey *et al.,* 2003). The precursor of vitamin D_3, 7-dehydrocholesterol is produced in relatively large quantities. 7-Dehydrocholesterol reacts with UVB light at wavelengths between 270 and 300 nm, with peak synthesis occurring between 295 and 297 nm (Hume *et al.,* 1927). These wavelengths are present in sunlight, as well as in the light emitted by the UV lamps in tanning beds (which produce ultraviolet primarily in the UVA spectrum, but typically produce 4% to 10% of the total UV emissions as UVB). Exposure to light through windows is insufficient because glass almost completely blocks UVB light (Claiborne, 2005; Bolton, 2013).

Adequate amounts of vitamin D can be produced with moderate sun exposure to the face, arms and legs, averaging 5–30 minutes twice per week, or approximately 25% of the time for minimal sunburn. The darker the skin, and the weaker the sunlight, the more minutes of exposure are needed. Vitamin D overdose is impossible from UV exposure; the skin reaches an equilibrium where the vitamin degrades as fast as it is created (Holick, 2007; Holick, 2002).

Sunscreen absorbs or reflects ultraviolet light and prevents much of it from reaching the skin (Holick *et al.,* 1987). Sunscreen with a sun protection factor (SPF) of 8 based on the UVB spectrum decreases vitamin D synthetic capacity by 95%, and SPF 15 decreases it by 98%.

The skin consists of two primary layers: the inner layer called the dermis, composed largely of connective tissue, and the outer, thinner epidermis. Thick epidermis in the soles and palms consists of five strata; from outer to inner, they are: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. Vitamin D is produced in the keratinocytes of two innermost strata, the stratum basale and stratum spinosum (Holick *et al.,* 1987).

2.5.16 Mechanism of metabolic activation of vitamin D

Vitamin D is carried in the bloodstream to the liver, where it is converted into the prohormone calcifediol. Circulating calcifediol may then be converted into calcitriol, the biologically active form of vitamin D, in the kidneys (Adams and Hewison, 2010).

Whether it is made in the skin or ingested, cholecalciferol is hydroxylated in the liver at position 25 (upper right of the molecule) to form 25-hydroxycholecalciferol (calcifediol or 25(OH)D). This reaction is catalyzed by the microsomal enzyme vitamin D 25-hydroxylase, the product of the *CYP2R1* human gene, and expressed by hepatocytes (Cheng *et al.,* 2004). Once made, the product is released into the plasma, where it is bound to an α-globulin carrier protein named the vitamin D-binding protein (Laing and Cooke, 2004).

Calcifediol is transported to the proximal tubules of the kidneys, where it is hydroxylated at the 1-α position (lower right of the molecule) to form calcitriol (1,25-dihydroxycholecalciferol, $1,25(OH)_2D$). The conversion of calcifediol to calcitriol is catalyzed by the enzyme 25-hydroxyvitamin D_3 1-alpha-hydroxylase, which is the product of the *CYP27B1* human gene. The activity of CYP27B1 is increased by parathyroid hormone, and also by low calcium or phosphate (Norman, 2008; Adams and Hewison, 2010).

Following the final converting step in the kidney, calcitriol is released into the circulation. By binding to vitamin D-binding protein, calcitriol is transported throughout the body, including to the classical target organs of intestine, kidney and bone. Calcitriol is the most potent natural ligand of the vitamin D receptor, which mediates most of the physiological actions of vitamin D (Norman, 2008; Adams and Hewison, 2010).

In addition to the kidneys, calcitriol is also synthesized by certain other cells including monocyte-macrophages in the immune system. When synthesized by monocyte-macrophages, calcitriol acts locally as a cytokine, modulating body defenses against microbial invaders by stimulating the innate immune system (Adams and Hewison, 2010).

2.5.17 Biological Activity of Vitamin D

The active vitamin D metabolite calcitriol mediates its biological effects by binding to the vitamin D receptor (VDR), which is principally located in the nuclei of target cells. The binding of calcitriol to the VDR allows the VDR to act as a transcription factor that modulates the gene expression of transport proteins (such as TRPV6 and calbindin), which are involved in calcium absorption in the intestine (Bouillon *et al.,* 2003). The vitamin D receptor belongs to the nuclear receptor superfamily of steroid/thyroid hormone receptors, and VDRs are expressed by cells in most organs, including the brain, heart, skin, gonads, prostate, and breast.

VDR activation in the intestine, bone, kidney, and parathyroid gland cells leads to the maintenance of calcium and phosphorus levels in the blood (with the assistance of parathyroid hormone and calcitonin) and to the maintenance of bone content (Insel *et al.,* 2015).

One of the most important roles of vitamin D is to maintain skeletal calcium balance by promoting calcium absorption in the intestines, promoting bone resorption by increasing

osteoclast number, maintaining calcium and phosphate levels for bone formation, and allowing proper functioning of parathyroid hormone to maintain serum calcium levels. Vitamin D deficiency can result in lower bone mineral density and an increased risk of reduced bone density (osteoporosis) or bone fracture because a lack of vitamin D alters mineral metabolism in the body. Thus, vitamin D is also critical for bone remodeling through its role as a potent stimulator of bone resorption (Bell *et al.,* 2010).

The VDR may be involved in cell proliferation and differentiation. Vitamin D also affects the immune system, and VDRs are expressed in several white blood cells, including monocytes and activated T and B cells (Watkins *et al.,* 2015). In vitro, vitamin D increases expression of the tyrosine hydroxylase gene in adrenal medullary cells, and affects the synthesis of neurotrophic factors, nitric oxide synthase, and glutathione (Puchacz *et al.,* 1996).

Apart from VDR activation, various alternative mechanisms of action are under study, such as inhibition of signal transduction by hedgehog, a hormone involved in morphogenesis (Sarkar *et al.,* 2010).

2.5.18 Guidelines for Dietary Recommendations

Different institutions propose different recommendations concerning daily amounts of the vitamin. The recommended daily intake of vitamin D *may* not be sufficient if sunlight exposure is limited (Glerup *et al.,* 2000).

(Conversion : 1 µg = 40 IU and 0.025 µg = 1 IU)

The European Menopause and Andropause Society recommends postmenopausal women consume15 µg (600 IU) until age 70, and 20 µg (800 IU) from age 71. This dose should be

increased to 100 µg (4,000 IU) in some patients with very low vitamin D status or in case of co-morbid conditions (Pérez-López *et al.,* 2012).

2.5.18.1 United State

According to the United States Institute of Medicine (Ross *et al..,* 2011) the recommended dietary allowances (RDA) of vitamin D are:

Age group	RDA (IU/day)
Infants 0–6 months	400*
Infants 6–12 months	400*
1–70 years	600 (15 µg/day)
71+ years	800 (20 µg/day)
Pregnant/Lactating	600 (15 µg/day)

- Asterisk for infants indicates adequate intake (AI) for infants, as an RDA has yet to be established for infants (Ross *et al.,* 2011)

2.5.19 Upper Intake Level of Vitamin D

The tolerable upper intake level (UL) is defined as "the highest average daily intake of a nutrient that is likely to pose no risk of adverse health effects for nearly all persons in the general population (Ross *et al.,* 2011). Although tolerable upper intake levels are believed to be safe, information on the long-term effects is incomplete and these levels of intake are not recommended (Ross *et al.* 2011). The dietary reference intake for vitamin D issued by the Institute of Medicine (IOM) in 2010 superseded a previous recommendation which had adequate

intake status. The recommendations were formed assuming the individual has no skin synthesis of vitamin D because of inadequate sun exposure. The reference intake for vitamin D refers to total intake from food, beverages and supplements, is intended for the North American population, and assumes that calcium requirements are being met (Ross *et al.,* 2011).

One school of thought contends the human physiology is fine-tuned to an intake of 4,000–12,000 IU/day from sun exposure with concomitant serum 25-hydroxyvitamin D levels of 40 to 80 ng/ml (Heaney and Holick, 2011) and this is required for optimal health. Proponents of this view, who include some members of the panel that drafted a now-superseded 1997 report on vitamin D from the IOM, contend the IOM's warning about serum concentrations above 50 ng/ml lacks biological plausibility. They suggest, for some people, reducing the risk of preventable disease requires a higher level of vitamin D than that recommended by the IOM (Heaney and Holick 2011*;* Holick *et al.,* 2010).

2.5.20 Allowable Health Claims

Apart from the above discussion on health effects or scientific evidence for lowering disease risk, governmental regulatory agencies stipulate for the food industry health claims allowable as statements on packaging.

European Food Safety Authority (EFSA) (EFSA*,* 2010)

1. normal function of the immune system
2. normal inflammatory response
3. normal muscle function
4. reduced risk of falling in people over age 60 (EFSA*,* 2011)

2.5.21 Dietary Sources of Vitamin D

Although vitamin D is not present naturally in most foods (Holick, 2006) it is commonly added as a fortification in manufactured foods, including some fruit juices and fruit juice drinks, meal replacement energy bars, soy protein-based beverages, certain cheese and cheese products, flour products, infant formulas, many breakfast cereals, and milk (de-Lourdes *et al.,* 2012; Spiro and Buttriss 2014).

While some studies have found that vitamin D_3 raises 25(OH)D blood levels faster and remains active in the body longer (Tripkovic, 2013; Alshahrani and Aljohani 2013) others contend that vitamin D_2 sources are equally bioavailable and effective as D_3 for raising and sustaining 25(OH)D (Keegan *et al.,* 2013; Biancuzzo *et al.,* 2013; Borel, *et al.,* 2015)

2.5.21.1 Mushrooms

Mushrooms can be a good dietary source of vitamin D_2 if exposed to ultraviolet light. Mushrooms contain high concentrations of ergosterol (provitamin D_2). Sunlight or ultraviolet radiation (UV) triggers conversion to viosterol (previtamin D_2), which then turns into vitamin D_2. Low values in mushrooms occur if there is little to no exposure to sunlight or UV light. When fresh mushrooms or dried powders are purposely exposed to artificial sunlight by use of an industrial ultraviolet lamp, vitamin D_2 levels can be concentrated to much higher levels (Keegan *et al.,* 2013; Simon *et al.,* 2013).

Human bioavailability of vitamin D_2 from vitamin D_2-enhanced button mushrooms via UV-B irradiation is effective in improving vitamin D status and not different from a vitamin D_2 supplement (Keegan *et al.,* 2013; Urbain *et al.,* 2011). Vitamin D_2 from UV-irradiated yeast

baked into bread or mushrooms is bioavailable and increases blood levels of 25(OH)D (Keegan *et al.,* 2013).

By visual assessment or using a chromometer, no significant discoloration of irradiated mushrooms, as measured by the degree of "whiteness", was observed (Koyyalamudi *et al.,* 2009)

Vitamin D_3 CHOLECALCIFEROL

In some countries, staple foods are artificially fortified with vitamin D (DRI, 1997). Natural sources include the following:

- Plant or fungal sources
    - Lichen
        - *Cladina arbuscula* specimens grown under different natural conditions: The contents of vitamin D_3 range from 0.67 to 2.04 μg g^{-1} dry matter in the thalli of *C. arbuscula* specimens grown under different natural conditions (Wang *et al.,* 2001)

2.5.21 Industrial Production of Vitamin D

Vitamin D_3 (cholecalciferol) is produced industrially by exposing 7-dehydrocholesterol to UVB light, followed by purification (Holick*,* 2005). The 7-dehydrocholesterol is a natural substance in fish organs, especially the liver (Takeuchi *et al.,* 1986) or in wool grease (lanolin) from sheep. Vitamin D_2 (ergocalciferol) is produced in a similar way using ergosterol from yeast or mushrooms as a starting material (Keegan *et al.,* 2013*;* Holick*,* 2005).

2.5.22 Effect of Heat on Vitamin D during Cooking

Vitamin D content in typical foods is reduced variably by cooking. Boiled, fried and baked foods retained 69–89% of original vitamin D (Jakobsen and Knuthsen, 2014).

2.5.23 Findings on Vitamin D

There is considerable research activity looking at effects of vitamin D and its metabolites in animal models, cell systems, gene expression studies, epidemiology and clinical therapeutics. These different types of studies can produce conflicting evidence as to the benefits of interventions with vitamin D (Dankers *et al.,* 2016).

Some preliminary studies link low vitamin D levels with disease later in life (Pyrżak *et al.,* 2015). One meta-analysis found a decrease in mortality in elderly people (Bjelakovic *et al.,* 2014). Another meta-analysis covering over 350,000 people concluded that vitamin D supplementation in unselected community-dwelling individuals does not reduce skeletal (total fracture) or non-skeletal outcomes (myocardial infarction, ischaemic heart disease, stroke, cerebrovascular disease, cancer) by more than 15%, and that further research trials with similar design are unlikely to change these conclusions (Bolland *et al.,* 2014).

Vitamin D deficiency is widespread in the European population (Cashman *et al.,* 2016). European research is assessing vitamin D intake levels in association with disease rates and policies of dietary recommendations, food fortification, vitamin D supplementation, and small amounts of sun exposure (Spiro and Buttriss, 2014).

2.6 Vitamin K

Vitamin K is a group of structurally similar, fat-soluble vitamins the human body requires for complete synthesis of certain proteins that are prerequisites for blood coagulation (K from *Koagulation*, Danish for "coagulation") and which the body also needs for controlling binding of calcium in bones and other tissues (LPI, 2014). The vitamin K-related modification of the proteins allows them to bind calcium ions, which they cannot do otherwise. Without vitamin K, blood coagulation is seriously impaired, and uncontrolled bleeding occurs. Preliminary clinical research indicates that deficiency of vitamin K may weaken bones, potentially leading to osteoporosis, and may promote calcification of arteries and other soft tissues (LPI, 2014)..

Chemically, the vitamin K family comprises 2-methyl-1,4-naphthoquinone (3-) derivatives. Vitamin K includes two natural vitamers: vitamin K_1 and vitamin K_2 (LPI, 2014). Vitamin K_2, in turn, consists of a number of related chemical subtypes, with differing lengths of carbon side chains made of isoprenoid groups of atoms.

Vitamin K_1, also known as phylloquinone, is made by plants, and is found in highest amounts in green leafy vegetables because it is directly involved in photosynthesis. It may be thought of as the plant form of vitamin K. It is active as a vitamin in animals and performs the classic functions of vitamin K, including its activity in the production of blood-clotting proteins. Animals may also convert it to vitamin K_2.

Bacteria in the gut flora can also convert K_1 into vitamin K_2 (menaquinone). In addition, bacteria typically lengthen the isoprenoid side chain of vitamin K_2 to produce a range of vitamin K_2 forms, most notably the MK-7 to MK-11 homologues of vitamin K_2. All forms of K_2 other than MK-4 can only be produced by bacteria, which use these forms in anaerobic respiration. The

MK-7 and other bacterially derived forms of vitamin K_2 exhibit vitamin K activity in animals, but MK-7's extra utility over MK-4, if any, is unclear and is a matter of investigation.

Because a synthetic form of vitamin K, vitamin K_3 (menadione), may be toxic by interfering with the function of glutathione, it is no longer used to treat vitamin K deficiency (LPI, 2014).

2.6.1 Health Effects of Osteoporosis

A review of 2014 concluded that there is positive evidence that monotherapy using MK-4, one of the forms of Vitamin K_2, reduces fracture incidence in post-menopausal women with osteoporosis, and suggested further research on the combined use of MK-4 with bisphosphonates. In contrast, an earlier review article of 2013 concluded that there is no good evidence that vitamin K supplementation helps prevent osteoporosis or fractures in postmenopausal women (Hamidi *et al.,* 2013).

A Cochrane systematic review of 2006 suggested that supplementation with Vitamin K_1 and with MK4 reduces bone loss; in particular, a strong effect of MK-4 on incident fractures among Japanese patients was emphasized (Cockayne *et al.,* 2006).

A review article of 2016 suggested to consider, as one of several measures for bone health, increasing the intake of foods rich in vitamins K_1 and K_2 (O'Keefe *et al.,* 2016).

2.6.2 Cardiovascular Health

Adequate intake of vitamin K is associated with the inhibition of arterial calcification and stiffening (Maresz*,* 2015), but there have been few interventional studies and no good evidence that vitamin K supplementation is of any benefit in the primary prevention of cardiovascular disease (Hartley *et al.,* 2015).

One 10-year population study, the Rotterdam Study, did show a clear and significant inverse relationship between the highest intake levels of menaquinone (mainly MK-4 from eggs and meat, and MK-8 and MK-9 from cheese) and cardiovascular disease and all-cause mortality in older men and women (Geleijnse *et al.,* 2004).

2.6.3 Cancer

Vitamin K has been promoted in supplement form with claims it can slow tumor growth; there is however no good medical evidence that supports such claims (Ades, 2009)

2.6.4 Warfarin Overdose and Coumadine Poisoning

Vitamin K is one of the treatments for bleeding events caused by overdose of the anticoagulant drug warfarin (Coumadin®) (Ageno *et al.,* 2012). Vitamin K is also part of the suggested treatment regime for poisoning by rodenticide (coumarin poisoning) (Lung, 2015).

2.6.5 Side Effects

Although allergic reaction from supplementation is possible, no known toxicity is associated with high doses of the phylloquinone (vitamin K_1) or menaquinone (vitamin K_2) forms of vitamin K, so no tolerable upper intake level (UL) has been set (Rasmussen *et al.,* 2006).

Blood clotting (coagulation) studies in humans using 45 mg per day of vitamin K_2 (as MK-4) (Ushiroyama *et al.,* 2002) and even up to 135 mg per day (45 mg three times daily) of K_2 (as MK-4) (Asakura *et al.,* 2001), showed no increase in blood clot risk. Even doses in rats as high as 250 mg/kg, body weight did not alter the tendency for blood-clot formation to occur (Ronden *et al.,* 1997).

Unlike the safe natural forms of vitamin K_1 and vitamin K_2 and their various isomers, a synthetic form of vitamin K, vitamin K_3 (menadione), is demonstrably toxic at high levels. The U.S. FDA has banned this form from over-the-counter sale in the United States because large doses have been shown to cause allergic reactions, hemolytic anemia, and cytotoxicity in liver cells (LPI, 2014).

2.6.6 Interactions

Phylloquinone (K_1) (Ansell *et al.,* 2004; Crowther, 2002) or menaquinone (K_2) are capable of reversing the anticoagulant activity of the anticoagulant warfarin (tradename Coumadin). Warfarin works by blocking recycling of vitamin K, so that the body and tissues have lower levels of active vitamin K, and thus a deficiency of vitamin K.

The newer anticoagulants dabigatran and rivaroxaban have different mechanisms of action that do not interact with vitamin K, and may be taken with supplementa0l vitamin K (Bauersachs *et al.,* 2010).

2.6.7 Chemistry

Vitamin K_2 (menaquinone). In menaquinone, the side chain is composed of a varying number of isoprenoid residues. The most common number of these residues is four, since animal enzymes normally produce menaquinone-4 from plant phylloquinone.

The three synthetic forms of vitamin K are vitamins K_3 (menadione), K_4, and K_5, which are used in many areas, including the pet food industry (vitamin K_3) and to inhibit fungal growth (vitamin K_5) (McGee*,* 2007).

2.6.8 Conversion of Vitamin K_1 to Vitamin K_2

Vitamin K_1 (phylloquinone) – both forms of the vitamin contain a functional naphthoquinone ring and an aliphatic side chain. Phylloquinone has a phytyl side chain.

The MK-4 form of vitamin K_2 is produced by conversion of vitamin K_1 in the testes, pancreas, and arterial walls (Shearer and Newman 2008) While major questions still surround the biochemical pathway for this transformation, the conversion is not dependent on gut bacteria, as it occurs in germ-free rats (Davidson *et al.,* 1998*;* Ronden *et al.,* 1998) and in parenterally-administered K_1 in rats (Thijssen *et al.,* 1994*;* Will *et al.,* 1992). In fact, tissues that accumulate high amounts of MK-4 have a remarkable capacity to convert up to 90% of the available K_1 into MK-4 (Davidson *et al.,* 1998*;* Ronden*, et al.,* 1998) There is evidence that the conversion proceeds by removal of the phytyl tail of K_1 to produce menadione as an intermediate, which is then condensed with an activated geranylgeranyl moiety to produce vitamin K_2 in the MK-4 (menatetrenone) form (Al-Rajabi*,* 2011).

2.6.9 Vitamin K_2

Vitamin K_2 (menaquinone) includes several subtypes. The two subtypes most studied are menaquinone-4 (menatetrenone, MK-4) and menaquinone-7 (MK-7).

2.6.10 Physiology

Vitamin K_1, the precursor of most vitamin K in nature, is a stereoisomer of phylloquinone, an important chemical in green plants, where it functions as an electron acceptor in photosystem I during photosynthesis. For this reason, vitamin K_1 is found in large quantities in the photosynthetic tissues of plants (green leaves, and dark green leafy vegetables such as romaine

lettuce, kale and spinach), but it occurs in far smaller quantities in other plant tissues (roots, fruits, etc.). Iceberg lettuce contains relatively little. The function of phylloquinone in plants appears to have no resemblance to its later metabolic and biochemical function (as "vitamin K") in animals, where it performs a completely different biochemical reaction.

Vitamin K (in animals) is involved in the carboxylation of certain glutamate residues in proteins to form gamma-carboxyglutamate (Gla) residues. The modified residues are often (but not always) situated within specific protein domains called Gla domains. Gla residues are usually involved in binding calcium, and are essential for the biological activity of all known Gla proteins (Furie *et al.,* 1999).

At this time, 17 human proteins with Gla domains have been discovered, and they play key roles in the regulation of three physiological processes:

1. **Blood coagulation**: Prothrombin (factor II), factors VII, IX, and X, and proteins C, S, and Z (Mann*,* 1999)
2. **Bone metabolism**: Osteocalcin, also called bone Gla protein (BGP), matrix Gla protein (MGP) (Price*,* 1988) periostin (Coutu *et al.,* 2008) and the recently discovered Gla-rich protein (GRP) (Viegas *et al.,* 2008*;* Viegas *et al.,* 2009)
3. **Vascular biology**: Growth arrest-specific protein 6 (Gas6) (Hafizi *et al.,* 2006)
4. **Unknown function**: Proline-rich γ-carboxyglutamyl proteins (PRGPs) 1 and 2, and transmembrane γ-carboxy glutamyl proteins (TMGs) 3 and 4 (Kulman *et al.,* 2007)

Like other lipid-soluble vitamins (A, D and E), vitamin K is stored in the fatty tissue of the human body.

2.6.11 Absorption and Dietary Needs

Previous theory held that dietary deficiency is extremely rare unless the small intestine was heavily damaged, resulting in malabsorption of the molecule. Another at-risk group for deficiency were those subject to decreased production of K_2 by normal intestinal microbiota, as seen in broad spectrum antibiotic use (Hafizi and Dahlbäck, 2006). Taking broad-spectrum antibiotics can reduce vitamin K production in the gut by nearly 74% in people compared with those not taking these antibiotics (Conly and Stein, 1994). Diets low in vitamin K also decrease the body's vitamin K concentration (Ferland *et al.,* 1993). Those with chronic kidney disease are at risk for vitamin K deficiency, as well as vitamin D deficiency, and particularly those with the apoE4 genotype (Holden *et al.,* 2010). Additionally, in the elderly there is a reduction in vitamin K_2 (Hodges *et al.,* 1990).

2.6.12 Deficiency of Vitamin K2

Average diets are usually not lacking in vitamin K, and primary deficiency is rare in healthy adults. Newborn infants are at an increased risk of deficiency. Other populations with an increased prevalence of vitamin K deficiency include those who suffer from liver damage or disease (e.g. alcoholics), cystic fibrosis, or inflammatory bowel diseases, or have recently had abdominal surgeries. Secondary vitamin K deficiency can occur in people with bulimia, those on stringent diets, and those taking anticoagulants. Symptoms of K_1 deficiency include anemia, bruising, nosebleeds and bleeding of the gums in both sexes, and heavy menstrual bleeding in women.

Osteoporosis (Ikeda *et al.,* 2006; Katsuyama *et al.,* 2002) and coronary heart disease (Sano *et al.,* 1999*;* Gast *et al.,* 2009) are strongly associated with lower levels of K_2 (menaquinone).

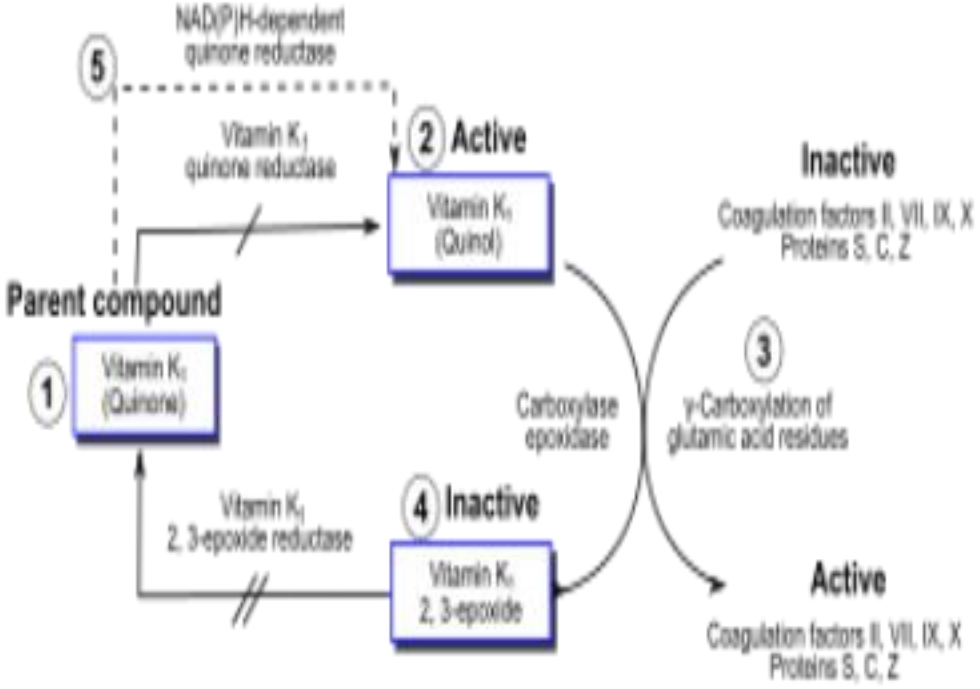


Figure 2.7: Mechanism of action of vitamin K_1.

2.6.13 Biochemistry Function in Animals

The function of vitamin K_2 in the animal cell is to add a carboxylic acid functional group to a glutamate (Glu) amino acid residue in a protein, to form a gamma-carboxyglutamate (Gla) residue. This is a somewhat uncommon posttranslational modification of the protein, which is then known as a "Gla protein". The presence of two −COOH (carboxylic acid) groups on the same carbon in the gamma-carboxyglutamate residue allows it to chelate calcium ions. The binding of calcium ions in this way very often triggers the function or binding of Gla-protein enzymes, such as the so-called vitamin K-dependent clotting factors discussed below.

Within the cell, vitamin K undergoes electron reduction to a reduced form called vitamin K hydroquinone, catalyzed by the enzyme vitamin K epoxide reductase (VKOR) (Oldenburg *et al.,* 2006) Another enzyme then oxidizes vitamin K hydroquinone to allow carboxylation of Glu to Gla; this enzyme is called gamma-glutamyl carboxylase (Suttie*,* 1985; Presnell *et al.,* 2002) or the vitamin K-dependent carboxylase. The carboxylation reaction only proceeds if the carboxylase enzyme is able to oxidize vitamin K hydroquinone to vitamin K epoxide at the same time. The carboxylation and epoxidation reactions are said to be coupled. Vitamin K epoxide is then reconverted to vitamin K by VKOR. The reduction and subsequent reoxidation of vitamin K coupled with carboxylation of Glu is called the vitamin K cycle (Stafford*,* 2005).

Warfarin and other 4-hydroxycoumarins block the action of VKOR (Whitlon *et al.,* 1978). This results in decreased concentrations of vitamin K and vitamin K hydroquinone in tissues, such that the carboxylation reaction catalyzed by the glutamyl carboxylase is inefficient. This results in the production of clotting factors with inadequate Gla. Without Gla on the amino termini of these factors, they no longer bind stably to the blood vessel endothelium and cannot activate clotting to allow formation of a clot during tissue injury. As it is impossible to predict what dose

of warfarin will give the desired degree of clotting suppression, warfarin treatment must be carefully monitored to avoid overdose.

2.6.14 Gamma-Carboxyglutamate Proteins

The following human Gla-containing proteins ("Gla proteins") have been characterized to the level of primary structure: blood coagulation factors II (prothrombin), VII, IX, and X, anticoagulant proteins C and S, and the factor X-targeting protein Z. The bone Gla protein osteocalcin, the calcification-inhibiting matrix Gla protein (MGP), the cell growth regulating growth arrest specific gene 6 protein (Gas6), and the four transmembrane Gla proteins (TMGPs), the function of which is at present unknown. Gas6 can function as a growth factor to activate the Axl receptor tyrosine kinase and stimulate cell proliferation or prevent apoptosis in some cells. In all cases in which their function was known, the presence of the Gla residues in these proteins turned out to be essential for functional activity.

Gla proteins are known to occur in a wide variety of vertebrates: mammals, birds, reptiles, and fish. The venom of a number of Australian snakes acts by activating the human blood-clotting system. In some cases, activation is accomplished by snake Gla-containing enzymes that bind to the endothelium of human blood vessels and catalyze the conversion of procoagulant clotting factors into activated ones, leading to unwanted and potentially deadly clotting.

Another interesting class of invertebrate Gla-containing proteins is synthesized by the fish-hunting snail *Conus geographus* (Terlau and Olivera, 2004). These snails produce a venom containing hundreds of neuroactive peptides, or conotoxins, which is sufficiently toxic to kill an adult human. Several of the conotoxins contain two to five Gla residues (Buczek *et al.,* 2005).

2.6.15 Methods of Vitamin K Assessment

1. The prothrombin time (PT) test measures the time required for blood to clot. A blood sample is mixed with citric acid and put in a fibrometer; delayed clot formation indicates a deficiency.
2. Undercarboxylated prothrombin (PIVKA-II); in a study of 53 newborns, found "PT (prothrombin time) is a less sensitive marker than PIVKA II" (Dituri *et al.,* 2012) and as indicated above, PT is unable to detect subclinical deficiencies that can be detected with PIVKA-II testing.
3. Plasma phylloquinone was found to be positively correlated with phylloquinone intake in elderly British women, but not men (Thane *et al.,* 2002*)*, but an article by Schurgers *et al.* reported no correlation between responses in a food frequency questionnaire and plasma phylloquinone (McKeown *et al.,* 2002)
4. Urinary γ-carboxyglutamic acid responds to changes in dietary vitamin K intake. Several days are required before any change can be observed. In a study by Booth *et al.*, increases of phylloquinone intakes from 100 μg to between 377 and 417 μg for five days did not induce a significant change. Response may be age-specific (Yamano *et al.,* 1989)
5. Undercarboxylated osteocalcin (UcOc) levels have been inversely correlated with stores of vitamin K (Matsumoto *et al.,* 2012) and bone strength in developing rat tibiae. Another study following 78 post-menopausal Korean women found a supplement regimen of vitamins K and D, and calcium, but not a regimen of vitamin D and calcium, was inversely correlated with reduced UcOc levels (Je *et al.,* 2011).

2.6.16 Function in Bacteria

Many bacteria, such as *Escherichia coli* found in the large intestine, can synthesize vitamin K_2 (menaquinone-7 or MK-7, up to MK-11) but not vitamin K_1 (phylloquinone) (Bentley and Meganathan, 1982). In these bacteria, menaquinone transfers two electrons between two different small molecules, during oxygen-independent metabolic energy production processes (anaerobic respiration) (Haddock and Jones, 1977). For example, a small molecule with an excess of electrons (also called an electron donor) such as lactate, formate, or NADH, with the help of an enzyme, passes two electrons to menaquinone. The menaquinone, with the help of another enzyme, then transfers these two electrons to a suitable oxidant, such fumarate or nitrate (also called an electron acceptor). Adding two electrons to fumarate or nitrate converts the molecule to succinate or nitrite plus water, respectively.

Some of these reactions generate a cellular energy source, ATP, in a manner similar to eukaryotic cell aerobic respiration, except the final electron acceptor is not molecular oxygen, but fumarate or nitrate. In aerobic respiration, the final oxidant is molecular oxygen (O_2), which accepts four electrons from an electron donor such as NADH to be converted to water. *E. coli*, as facultative anaerobes, can carry out both aerobic respiration and menaquinone-mediated anaerobic respiration.

2.6.17 Injection in New Born

The blood clotting factors of newborn babies are roughly 30–60% that of adult values; this may be due to the reduced synthesis of precursor proteins and the sterility of their guts. Human milk contains 1–4 μg/L of vitamin K_1, while formula-derived milk can contain up to 100 μg/L in supplemented formulas. Vitamin K_2 concentrations in human milk appear to be much lower than

those of vitamin K_1. Occurrence of vitamin K deficiency bleeding in the first week of the infant's life is estimated at 0.25–1.7%, with a prevalence of 2–10 cases per 100,000 births (Shearer, 1995). Premature babies have even lower levels of the vitamin, so they are at a higher risk from this deficiency.

2.6.17.1 United State

As a result of the occurrences of vitamin K deficiency bleeding, the Committee on Nutrition of the American Academy of Pediatrics has recommended 0.5–1 mg of vitamin K_1 be administered to all newborns shortly after birth (AAPCFN, 2003).

2.6.17.2 United Kingdom

In the UK vitamin K supplementation is recommended for all newborns within the first 24 hours (Logan and Gilbert, 1998). This is usually given as a single intramuscular injection of 1 mg shortly after birth but as a second-line option can be given by three oral doses over the first month.

2.6.18 Controversy

Controversy arose in the early 1990s regarding this practice, when two studies suggested a relationship between parenteral administration of vitamin K and childhood cancer (Parker *et al.,* 1998), after treating children for serious bleeding problems. They cited lack of newborn vitamin K administration as the reason why the problems occurred, and recommended that breastfed babies could have an increased risk unless they receive a preventative dose.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study Area

This research was carried out at Federal Medical Centre, Owo, Ondo State. Nigeria. The Hospital serves an estimated population of 509,000 and also serves as a reference center for orthopedic and dissibilities. It also covers a large geographical area boarding the northern part with an estimated area of 6353 $km^2$2453 square miles with an estimated population of 2,737,186. Federal Medical Center was upgraded in 2006 to serve as a referral hospital for treatment and management of subjects with dissibility. The hospital is currently serving neighboring states such as Ondo, Ekiti, Osun State and some parts of Edo.

3.2 Cross Sectional Study

A cross-sectional random sampling method was used for collection of (300) Blood samples comprizing of One Hundred and fifty (150) osteoarthritis patients and One Hundred and fifty (150) blood sample of non osteoarthritis patients. The samples were collected from the blood bank of screened patient following all legal and professional ethical documentations. Blood samples were collected into 5ml capacity plain plastic bottles with the help of health personnel in the hospital.

3.2.1 Inclusion Criteria

All patients included were aged 50 and above with knee injury, Hip OA, non smoker and non alchoholic patients.

3.2.2 Exclusion Criteria

1. All patients excluded had Inflammatory arthritis, Uncontrolled DM, HTN, CKD and uncorrected Hypo/hyperthyroidism

3.3 Ethical Approval

An Ethical clearance was obtained from the Research and Ethical Committee of Federal Medical Centre, Owo. Also, written informed concent was sort from the participants as well as giving assurance the health history of the patients obtained will not in any way be linked with the true identity of the patient when recording the outcome of my findings.

3.4 Sample Size Determination

Sample size will be determined according to the method of Daniel *et al*., (1999).

$$n = \frac{Z^2 P(1- P)}{d^2}$$

Where,

n = sample size

P = prevalence rate in percentage

Z = confidence interval of 95% which is equivalent to confidence coefficientn of 1.96

d = desired level of precision or significance which is equal to 0.05.

The prevalence rate of knee osteoarthritis in Nigeria is 8.9% (Aderonke *et al*., 2007)

$$N = \frac{(1.96)^2 \text{ x } 0.89 \text{ x } (1-0.89)}{(0.05)^2}$$

$$N = \frac{3.8416 \times 0.89 \times 0.11}{0.0025}$$

$$N = \frac{0.376}{0.0025}$$

$N = 150$ this is the number of sample size.

In this study a total of 150 osteoarthritis samples were used.

3.5 Sample Preservation

The blood sample collected were immediately stored in the thermo-flask containing ice pack for transportation to Central Research Laboratory, Federal University of Technology, Akure where the analysis were conducted.

3.6 Sample Collection

The whole blood sample collected was centrifuged using refrigerated centrifuge manufactured by Harrier, Model 18/80 at speed 10000 rpm for 15mins. The distinct layer obtained i.e. Plasma and Serum where plasma were kept at low temperature for vitamin D analysis. The blood serum intended to use for analysis were stored under a low temperature of about -45^{o}C.

3.6.1 Procedure for analysis of zinc in blood serum

3.6.1.1 Analysis of zinc and sample preparation

500µL of serum collected were mixed with 2N HCl into a 5ml Volumetric flask with the aid of graduated dispensing bottle and left for 24hrs.The mixture was centrifuged at 4000rpm for 15 minutes with the use of bench top centrifuge Harrier 18/80 model.

The supernatant obtained was analyzed for different elements using Atomic Absorption Spectrometer VGP210 model manufactured by Buck Scientific, USA

The machine readings obtained was used to calculate the amount of zinc present in the serum in mg/l using the equation below

$$\text{Conc of Zn in (ug/ml)} = \frac{\text{Total Volume of Samples} * \text{Machine readings obtained}}{\text{Volume of Serum Used}}$$

3.6.1.2 Digestion Analysis of sample for Ca

The blood sample will be digested using a modified method of (Yahaya *et al.,* 2013). The Blood samples were digested by the Conventional Wet Acid Method by Measuring 2mL of blood serum into Pyrex flask and 3 mL of freshly prepared mixture of concentrated nitric acid and hydrogen peroxide [HNO_3 :H_2O_2) and allow to stand for 10 minutes. The flasks were covered with watched glass and then digested at 60 - 70^{0}C for 1 - 2 hours. The digests will then be treated with 2 mL of nitric acid and few drops of H_2O_2, while heating continuously in hot plate at about 80^{0}C until a clear digested solution was obtained. The excess acid mixture will be evaporated to semi - dry mass, cooled and diluted with 0.1 mL nitric acid and will later be transferred to 100 mL volumetric flask and diluted to mark using doubled distilled-deionized water. A blank extraction (without the sample) were also carried out through the complete procedure using doubled distilled-deionized water. The digest will then be analyzed using Atomic Absorption Spectrophotometer Manufactured by Buck Scientific, Model VGP 210 located at Central Research Laboratory, Federal University of Technology, Akure, Ondo State.

3.6.1.3 Atomic absorption spectrometer conditions for the analysis

Lamp Type Used:	Hollow Cathode Lamp
Fuel Used:	Acetylene/Air
Slit:	1.8

3.6.1.4 Measurement of Metals

The trace elements were assayed using atomic absorption spectroscopy (Buck Scientific Model VGP-210, Germany) at the federal university of science and technologhy Akure, Ondo State. The metal content of the digested samples were determined by aspiration (air/acetylene flame) using an atomic absorption spectroscopy (Buck Scientific Model VGP-210, Germany) and appropriate wavelengths were selecteed for different trace elements. Their concentrations were obtained in duplicate from the absorbance read.

Quality control and assurance- replicate analyses were performed on samples to yield a mean which was used to determine trueness and also standard deviation of the mean to measure precision (AOAC, 1990). Procedural blanks and standard solutions were also included for analytical quality control to assure the accuracy.

3.6.1.5 Principle of atomic absorption spectroscopy (AAS)

Atomic absorption spectroscopy is based on the principle that when a beam of electromagnetic radiation passed through a substance, the radiation may either be absorbed, or transmitted depending upon the wavelength of the radiation. The absorption of radiation would bring about an increase in the energy of the molecule. The energy gained by the molecule is directly proportional to the wavelength of radiation. The increase in the energy of the molecule leads to

the electronic excitation where electron jumps to higher ground levels. A particular wavelength that a given molecule can absorb depends upon the change in vibrational, rotational or electronic states of the subject samples.

3.6.1.6 Determination of vitamin K

The procedure for colour development as adopted from Menotti's procedure is as follows. The solution in which the concentration of vitamin K is being determined is placed in a flask and the sodium pentacyanoamineferroate reagent is added. The solution was then stirred and then allowed to stand for fifteen minutes to allow maximum colour development. When the blue color has developed, the absorption of the solution is measured by means of a spectrophotometer at 650nm.The standard vitamin K solution was prepared by dissolving 5 milligrams of crystalline vitamin K in water and diluting to 100 milliliters. This solution is stable for 4 to 6 hours. The absorption of the solution was read on a spectrophotometer at 650nm, against a reagent blank.

3.6.1.7 Sample preparation of Vitamin D

3.6.1.7.1 Analysis using HPLC

The extracted sample was analyzed for Vitamin D1 and D2 metabolites using C_{18} column and mobile phase Water: Methanol and 0.1% Formic acid in the two solvents. The reference standard will be purchased through Sigma Aldrich with purity of 99.99%.

Vitamin D metabolites were extracted with a liquid-liquid extraction method. Plasma (150 µL) were mixed with 0.2M ZnSO4 (150 µL) in a 2-mL glass HPLC vial and 300 µL of methanol containing 25ng/mL of d 6- 25(OH)D3 (internal standard) and vortex were mixed (10secs). 750 µL of hexane were added and mixed for 30secs, which were then centrifuged for 10mins at

4000rpm. The hexane layer (650μL) was removed and placed into the micro-vial and evaporated to dryness under nitrogen at 55°C. The dried extract was reconstituted with 75μL of 15:85 water, methanol solution and injected (5 μL) for analysis.

3.7 Statistical Analysis

All data obtained were subjected to SPSS Version 16.0 statistical analysis using the chi-square and students't-test. Data was significant at $P \leq 0.05$.

CHAPTER FOUR

RESULTS

Table 4.1 compares the socio-demographics characteristics of study participants which shows absence of significant difference when measured variable are compared accros all age group. The same was obtained between male and female subjects (P > 0.372 and 0.557 respectively). Osteoartthristis were more in Female than Male Subjects with a percentage difference of 60.7% and 39.3% respectivbely. Our research however showed that highe portion of subjects within the age bracket of 61-70 years age bracket had 56.0% of of Osteoarthritis, followed by those within the age bracket of 51- 60 years of age. The least proportion were 70 - 80 years old with 5.3%.

Table 4.2 shows the result of age and gender compared between Osteoarthritis subject and non Osteoarthritis patients. the age bracket lower than 65 years had 46(30.7%) while the age bracket greater than 65 years had 104 (69.3%) out of 150 subject tested in this work. the test subject was compared with control and there was no significant difference.

the mean age of the subject was also tested with t test and there were no significant difference connecting age and Osteoarthritis. The t test shows that Osteoarthritis is independent of age within the age bracket of study.

(*t – Independent test)*

But the preponderance of Osteoarthritis were highly recorded in female than male subject which had 91 (60.7%) and 59 (39.3%) respectively.

Table 4.1: Socio-demographic characteristics study population of Osteoarthritis and Non Osteoarthritis patents

Age (years)	Case n (%) N = 150	Control n (%) N = 150	Chi square	p-value
51 – 60	30 (20.0)	28 (18.7)	3.132	0.372
61 – 70	84 (56.0)	88 (58.7)		
71 – 80	8 (5.3)	14 (9.3)		
81 – 90	28 (18.7)	20 (13.3)		
Gender				
Male	59 (39.3)	64 (42.7)	0.344	0.557
Female	91 (60.7)	86 (57.3)		

Table 4.2: Age and Gender compared between Osteoarthritis and Non Osteoarthritis patents

Variable	Cases (%) N = 150	Control (%) N = 150	Chi square	p-value
Age Group (in years)				
< 65	46 (30.7)	54 (36.0)	0.960	0.327
≥ 65	104 (69.3)	96 (64.0)		
Mean age ± SD	*68.3 ± 9.2*	*68.2 ± 7.6*	*0.137[t]*	*0.891*
Gender				
Male	59 (39.3)	60 (60.0)	0.906	1.000
Female	91 (60.7)	90 (60.0)		

t – Independent test

Table 4.3 shows measured mineral concentration of osteoarthritis and non osteoarthritis subjects showing that calcium, copper and selenium were statistically significant between there distributions ($P < 0.001$). Zinc however was not significant different ($p = 0.121$). There were 61.3% with Low Calcium Concentaration and 38.7% Subjects with Normal Calcium concentration among the Osteoathritis subjects while in Non Osteoarthritis subjects 26.7% had low and 73.3% had normal Calcium concentration. In copper, there were 75.3% of subjects with Low copper and 24.7% of Osteoarthritis subjects having a normal copper concetration, while the Non-osteoarthritis subjects had 46.0% and 54.0% for both Low and Normal copper contration. the Other mineral of importance such as zink and selenium had also shown to have 20.0% , 80.0% (low, normal) with the Non Osteoarthritis subjects having 13.3 and 86.7% low and Normal Zink concentration. But in selenium Concentrations, this work recorded 16.7%, 72.7% and 10.7% with low, Normal and High selenium concentrations values respectively. while the Non-osteoarthritis subjects had 5.3%, 66.0% and 28.7% with low, Normal and High values of selenium concentrations respectively.

Table 4.4: Shows the Measured parameters of Osteoarthritis subjects compared with Non Osteoarthritis. Result generated from the analysis showed decreased significant difference between Calcium, Copper, Zinc, Selenium, Vitamin K of Osteoarthritis subject when compared with Non Osteoarthritis patients except Vitamin D which result generated from t-test showed absence of significant difference.

Table 4.3: Shows the proportion of subjects with Osteoarthritis and Non Osteoarthritis subjects who had low and normal masured variable based on locally determined reference range

Variable	Case n (%) N = 150	Control n (%) N = 150	Chi square	p-value
Calcium (mmol/L)			36.580	**<0.001**
Low (< (2.2)	92 (61.3)	40 (26.7)		
Normal (2.2 -2.6)	58 (38.7)	110 (73.3)		
Copper (µm/L))			27.044	**<0.001**
Low (< 11)	113 (75.3)	69 (46.0)		
Normal (11- 22)	37 (24.7)	81 (54.0)		
Zinc (µm/L)			2.400	0.121
Low (<11.5)	30 (20.0)	20 (13.3)		
Normal (11.5- 18.5)	120 (80.0)	130 (86.7)		
Selenium (ng/mL)			21.594	**<0.001**
Low (< 70)	25 (16.7)	8 (5.3)		
Normal (70 -150)	109 (72.7)	99 (66.0)		
High (>150)	16 (10.7)	43 (28.7)		

Table 4.4: Measured parameters compared between Osteoarthritis and Non Osteoarthritis Subjects

Variable	Subject Mean ± SD N = 150	Control Mean ± SD N = 150	t test	p-value
Calcium	1.86 ± 0.23	1.76 ± 0.30	3.462	**0.001**
Copper	8.69 ± 3.77	9.84 ± 4.34	-2.452	**0.015**
Zinc	12.71 ± 1.95	13.61 ± 2.34	-3.644	**<0.001**
Selenium	141.13 ± 53.86	106.07 ± 36.93	6.576	**<0.001**
Vit K	1.60 ± 0.25	1.67 ± 0.32	-2.291	**0.023**
Vit D	54.59 ± 15.69	53.74 ± 16.12	0.461	0.645

Table 4.5: Present measured parameters of Osteoarthritis subjects compared with age groups of non-osteoarthritis subjects. Result generated from the data showed decreased significant difference between Calcium, Copper, Zinc, Selenium, except Vitamin K and and Vitamin D which were not significant.

Table 4.6: Present measured parameters of Osteoarthritis subjects compared with genders of non-osteoarthritis subjects. Result generated from the analysis showed a significant difference between Calcium, Copper, Zinc, and Vitamin D, except Selenium and Vitamin K which were not significant.

Table 4.7 compares the levels of vitamins D and K between the Osteoarthritis and Non-osteoarthritis control groups. About 19.3% of the cases with osteoarthritis had a low level of Vitamin K compared to only 9.3% of the apparently healthy controls. This difference was found to be statistically significant ($p = 0.013$). Similarly, 30.7% casas with osteoarthritis subjects had low level of Vitamin D and 5.3% among Non-osteoarthritis had low level of vitamin D ($P < 0.001$).

Means of all measured parameters in table 4.8 were compared with the control groups and this were found to be statistically significant different at 0.05 level except age which shows a very high significant difference ($P < 0.116$) between Osteoarthritis and Non-osteoarthritis subjects.

Table 4.5: Measured parameters compared between the age groups of Osteoarthritis and Non Osteoarthritis patents

Variable	<65 years Mean ± SD N = 100	≥ 65 years Mean ± SD N = 200	t test	p-value
Calcium	1.69 ± 0.30	1.87 ± 0.23	-6.070	**<0.001**
Copper	6.20 ± 2.54	10.79 ± 3.87	-10.746	**<0.001**
Zinc	12.61 ± 2.26	13.44 ± 2.12	-3.145	**0.002**
Selenium	102.50 ± 2.26	134.15 ± 37.70	-5.487	**<0.001**
Vit K	1.65 ± 0.22	1.63 ± 0.32	0.733	0.464
Vit D	54.45 ± 17.51	54.03 ± 15.05	0.216	0.829

Table 4.6: Measured parameters compared between the genders of Osteoarthritis and Non Osteoarthritis patents

Variable	Male Mean ± SD N = 119	Female Mean ± SD N = 181	t test	p-value
Calcium	1.76 ± 0.31	1.84 ± 0.23	-2.516	**0.012**
Copper	8.65 ± 4.36	9.66 ± 3.88	-2.101	**0.037**
Zinc	12.65 ± 1.96	13.50 ± 2.29	-3.301	**0.001**
Selenium	118.15 ± 48.31	127.18 ± 49.80	-1.555	0.121
Vit K	1.65 ± 0.33	1.62 ± 0.26	0.723	0.470
Vit D	47.58 ± 15.87	58.50 ± 14.37	-6.178	**<0.001**

Table 4.7: The concentration of vitamin D and K in Osteoarthritis and Non Osteoarthritis patients

Variable	Case n (%) N = 150	Control n (%) N = 150	Chi square	p-value
Vitamin K (mm/L)			6.108	**0.013**
Low	29 (19.3)	14 (9.3)		
Normal	121 (80.7)	136 (90.7)		
Vitamin D (ng/L)			32.611	**<0.001**
Low	46 (30.7)	8 (5.3)		
Normal	104 (69.3)	142 (94.7)		

Table 4.8: Mean comparisons of data obtained from Osteoarthritis and Non Osteoarthritis patients

Variable	Case Mean ± SD N = 150	Control Mean ± SD N = 150	t-test	p-value
Age (years)	68.3 ± 9.2	66.7 ± 8.5	1.574	0.116
Calcium (mmol/L)	2.1 ± 0.2	2.3 ± 0.3	6.398	**<0.001**
Copper (mmol/L)	8.7 ± 3.8	12.0 ± 4.3	7.138	**<0.001**
Zinc (μm/L)	12.7 ± 1.9	13.6 ± 2.3	3.780	**<0.001**
Selenium (ng/mL)	109.4 ± 38.2	136.1 ± 36.9	6.144	**<0.001**
Vitamin K (mm/L)	0.61 ± 0.26	0.72 ±0.32	3.246	**0.001**
Vitamin D (ng/L)	30.2 ± 14.9	53.7 ± 16.1	13.148	**<0.001**

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Osteoarthritis is a chronic progressive degenerative disorder of synovial joints affecting both the articular cartilage and the underlying subchondral bone (Howell *et al.,* 1982). Although most researchers are focused on the articular cartilage in order to identify the earliest changes in OA (Bland, 1983), few studies have concentrated their efforts on the functional status and periodic measurement of some trace elements that help in the lubrication of vital joint ensuring holistic care and maintenance of the elderly subjects. In this study, evaluation of serum levels of some trace elements calcium, zinc, copper, selenium, Vitamin D and K in patients diagnosed with osteoarthritis among elderly attendees at a primary care clinic were reported. Information generated from the socio-demographic distributions of the study population depicts no significant difference when measured values were compared across all age groups, the same was obtained in the measurement between male and female ($P < 0.372$ and 0.557 respectively). the preponderance of Osteoarthritis was highly recorded in female than male subject which had 91 (60.7%) and 59 (39.3%) respectively.

More osteoarthritis subjects were found within the age bracket of 61 - 70 years age bracket representing 56.0%, followed by those within the age bracket of 51- 60 years. The least were those in 70 - 80 years old with 5.3%. The higher prevalence of physical disability for the basal activity of daily living (BADL) components amongst elderly women compared to elderly men in this study have been reported in previous studies (Tas *et al.,* 2007; Tas *et al.,* 2007).

Chappell and Cooke had previoulsly opined, that the increasing trend of functional disability in elderly patients might be due to the increasing prevalence of chronic illnesses as ageing sets in (Chappell and Cooke, 2012).

Result generated from the age and gender of Osteoarthritis subject and non Osteoarthritis patients, showed that the age bracket lower than 65 years had 46(30.7%) while the age bracket greater than 65 years had 104 (69.3%) out of 150 subject tested in this work. the test subject was compared with control and there were absence of significant difference. The t test shows that Osteoarthritis is independent of age within the age bracket of study. (*t – Independent test)* the mean age of the subject was also tested with t test and there were no significant difference connecting age and Osteoarthritis among our study population. Out side the age, this kind of result sugesting presence of endothelial injury which might result from oxidative damage of the amino acide needed for tissue lubrication, repair, cell signal and growth (Fu *et al*., 2008). Similarly, the concentration of calcium, copper and selenium were statistically significant between other measured distributions ($P \leq 0.001$). Zinc however showed no significant difference ($P \geq 0.121$) among osteoarthritis and non osteoarthritis subjects as seen in this study. Naturally occurring minerals such as zinc (Zn) copper (Cu), and selenium (Se) have shown anti-Inflammatory effects in both animal and human studies. Animal model of OA, Copper is an essential cofactor in enzymes such as super oxide dismutase (SOD) that also needs Zn as cofactors. Many studies revealed a role for oxidative stress in the pathogenesis of OA, whereby ROS generation and impaired antioxidant status of the joint might result in the degradation of cartilage joint remodeling (Henrotin *et al*., 2005). Roughly 2 - 4grams of zinc (Rink and Gabriel, 2000) are distributed throughout the human body. Most zinc are found in the brain, muscles, bones, kidney, and liver, with the highest concentrations in the prostate and parts of the eye

(Wapnir, 1990). Semen in particular is rich in zinc and is a key factor in prostate gland function and reproductive organ growth (Berdanier *et al.,* 2007). Zinc homeostasis also plays a critical role in the functional regulation of the central nervous system (Prakash *et al.,* 2015*;* Cherasse and Urade, 2017). Dysregulation of zinc homeostasis in the central nervous system results in excessive synaptic zinc concentrations and is believed to induce neurotoxicity resulting in mitochondrial oxidative stress (e.g., by disrupting certain enzymes involved in the electron transport chain, including complex I, complex III, and α-ketoglutarate dehydrogenase), the dysregulation of calcium homeostasis, glutamatergic neuronal excitotoxicity, and interference with intraneuronal signal transduction (Tyszka *et al.,* 2014; Prakash *et al.,* 2015).

The parameters Measured among Osteoarthritis subjects was compared with Non Osteoarthritis patients and there were decreased significant difference between Calcium, Copper , Zinc, Selenium, VitK of Osteoarthritis subject when compared with Non Osteoarthritis patients. except VitD which result generated from t-test showed absence of significant difference. A decreased significant diffrence of Calcium, Copper , Zinc, Selenium, was also observed accros the age groups of Osteoarthritis and non-Osteoarthritis subjects, except VitK and and VitD which were not significant. Similarly the Osteoarthritis subjects compared with genders of non Osteoarthritis subjects showed a significant difference between Calcium, Copper , Zinc, and Vit D, except Selenium and VitK which were not significant.

it was suggested by Shin *et al*., (2011) that reduced serum levels of trace element might expose individual to damages mediated through oxidative stress. One of the pathobiological consequences of reduced serum levels of trace element as shown from this study might result to injury resulting from oxidative damage of the proteins needed in tissue lubrication, which may lead to altered cell signal, repair, growth which might result to ROS. Oxidative stress and the

cellular anti-oxidant defense system are regulated in a coordinated fashion during inflammation. It is known that reactive oxygen species (ROS) such as hydrogen peroxide and superoxides are released and scavenged during wound healing (Prosser *et al*., 2006). Glutathione is another prominent player in the cellular antioxidant function (Lu, 2013; Espinosa-Diez et al., 2015) in increasing glutathione suppresses Hepatic stellate cells (HSCs) growth and activation (Fu *et al*., 2008). it also stimulate TGF-β and also suppressed expression of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione biosynthesis, (Fu *et al*., 2008).

Zinc deficiency is usually due to reduced or insufficient dietary intake, but can be associated with malabsorption, acrodermatitis enteropathica, chronic liver disease, chronic renal disease, sickle cell disease, diabetes, malignancy, and other chronic illnesses (Prasad, 2003). Groups at risk for zinc deficiency include the elderly.

Selenium is also an essential co-factor for glutathione peroxidase which may have a role in reducing the incidence of osteoarthritic lesion (Kurz *et al*., 2002). Demonstrating lower concentrations of Zn, Se and higher concentrations of Cu and Cu/Zn ratio in patients with knee OA compared with controls; in addition to strong association of disease duration and severity with serum concentrations of Cu, Zn and Se. However, Selenium is an important constituent of glutathione peroxidase enzyme and its deficiency resulting in a marked decline in glutathione peroxidase activity of many tissues, which leads to increased oxidative stress (Shenkin, 2009).

There were 61.3% of subjects with Low calcium concentration and 38.7% subjects with normal calcium concentration among the osteoarthritis subjects while the non osteoarthritis subjects, 26.7% had normal and 73.3% had low calcium concentration. 75.3% of subjects had low copper concentration and 24.7% of osteoarthritis subjects with normal copper concentration, while the non osteoarthritis subjects had 46.0% and 54.0% for both low and normal copper concentration.

The other important minerals such as zinc and selenium had also shown to have 20.0% and 80.0% (low and normal zinc concentration) while the non osteoarthritis subjects had 13.3% and 86.7% low and normal zinc concentration respectively. But the selenium concentration levels recorded in this work indicate that 16.7%, 72.7% and 10.7% represent low, normal and high selenium concentrations respectively for osteoarthritis. While the non osteoarthritis subjects had 5.3%, 66.0% and 28.7% representing number of subjects with low, normal and high values of selenium concentrations respectively. In blood plasma, zinc is bound to albumin and about (60%, low-affinity) are transported as transferrin representing (10%) in the whole blood (Wapnir, 1990). Transferrin also transports iron, excessive iron reduces zinc absorption, and vice versa. A similar antagonism exists with copper (Whitney *et al.,* 2005). The human dopamine transporter contains a high affinity extracellular zinc binding site which, upon zinc binding, inhibits dopamine reuptake and amplifies amphetamine-induced dopamine efflux *in vitro* (Krause, 2008; Sulzer, 2011; Scholze *et al.,* 2002). In the present study, serum Zn and Se concentrations were as low as 20.0% and 16.7%.

It is not known whether trace element status leads to disease or whether diseases set in due to the deficiency of trace elements. Although, Oxidative damage to essential cell components caused by oxygen free radicals results in the pathobiology of degenerative joint disease (Haskin *et al*., 1995). It is generally believed that a strict metabolic control delays the development of late complications in OA. A relationship was observed between OA and trace elements in many research studies. In many cases, changes in the concentration of these elements were demonstrated.

According to some other authors, this increased blood serum Cu concentration was even considered to be a marker of clinical activity of this disease (Milanino *et al*., 1993). Decreased

levels of Se and the activity of selenium-dependent enzymes have also been studied in other diseases, including epilepsy, which showed a strong correlation between their reduction and severity of the disease (Shams *et al*., 2007). Decreased serum Se levels in humans is unlikely to happen, but may be the etiological factor of some serious disorders such as Keshan disease (endemic cardiomyopathy) and Kashin-Beck disease (endemic osteoarthritis). Due to absence of some variables, including body mass index (BMI), smoking, dietary intakes and use of anti-inflammatory agents, potential confounding biases could not be excluded. However, some study did not find any association between drug doses, and smoking status, and the changes of several trace elements, including Se, Fe, Cu and Zn (Yazar *et al*., 2005). Understanding that Se is a cofactor of some enzymes with antioxidant activity; we can suggest that reduced serum levels of this element renders the individual prone to various damages mediated through oxidative stress (Shin *et al*., 2011).

The present study also has some significant advantages. The first strength is the simultaneous estimation of 4 trace elements and 2 Vitamines under the same experimental conditions.

Table 4.7 compares the levels of vitamins D and K between the Osteoarthritis and Non Osteoarthritis control groups. About 19.3% of the cases with osteoarthritis had a low level of Vitamin K compared to 9.3% of non osteoarthritis controls subjects. This difference was found to be statistically significant ($P < 0.013$). Also, about 30.7% and 5.3% respectively of the Osteoarthritis and Non Osteoarthritis had a low level of vitamin D. Vitamin D has a significant role in calcium homeostasis and metabolism. Its discovery was due to effort to find the dietary substance lacking in children with rickets (the childhood form of osteomalacia) (Wolf, 2004) Vitamin D supplements are given to treat or to prevent osteomalacia and rickets, but the evidence for other health effects of vitamin D supplementation in the general population is inconsistent

(Pittas *et al.,* 2010; Chiung *et al.,* 2009). The effect of vitamin D supplementation on mortality is not clear, with one meta-analysis finding a small decrease in mortality in elderly people (Bjelakovic *et al.,* 2014) and another concluding no clear justification exists for recommending supplementation for preventing many diseases, and that further research of similar design is not needed in these areas (Boliland *et al.,* 2014). However, vitamin D deficiency has become a worldwide problem in the elderly and remains common in children and adults (Eriksen and Glerup 2002*;* Holick*,* 2007) Low blood calcifediol (25-hydroxy-vitamin D) can result from avoiding the sun (Schoenmakers *et al.,* 2008) Deficiency results in impaired bone mineralization and bone damage which leads to bone-softening diseases (Grant *et al.,* 2005*;* Brown *et al.,* 2013) including rickets, osteomalacia and osteoathritis.

For older people with osteoporosis, taking vitamin D with calcium may help prevent hip fractures, but it also slightly increases the risk of stomach and kidney problems (Avenell *et al.,* 2014*)*. Supplementation with higher doses of vitamin D, in those older than 65 years, may decrease fracture risk (Bischoff-Ferrari *et al.,* 2012). The effect may be smaller for people living independently than for people in institutions (Chung *et al.,* 2011). Calcium regulation in the human body (Boron *et al.,* 2016). The role of active vitamin D (1,25-dihydroxyvitamin D, calcitriol) is shown in orange. The active vitamin D metabolite calcitriol mediates its biological effects by binding to the vitamin D receptor (VDR), which is principally located in the nuclei of target cells. The binding of calcitriol to the VDR allows the VDR to act as a transcription factor that modulates the gene_expression of transport proteins (such as TRPV6 and calbindin), which are involved in calcium absorption in the intestine (Bouillon *et al.,* 2003). The vitamin D receptor belongs to the nuclear receptor superfamily of steroid/thyroid hormone receptors, and

VDRs are expressed by cells in most organs, including the brain, heart, skin, gonads, prostate, and breast.

VDR activation in the intestine, bone, kidney, and parathyroid gland cells leads to the maintenance of calcium and phosphorus levels in the blood (with the assistance of parathyroid hormone and calcitonin) and to the maintenance of bone content (Insel *et al.,* 2015). One of the most important roles of vitamin D is to maintain skeletal calcium balance by promoting calcium absorption in the intestines, promoting bone resorption by increasing osteoclast number, maintaining calcium and phosphate levels for bone formation, and allowing proper functioning of parathyroid hormone to maintain serum calcium levels. Vitamin D deficiency can result in lower bone mineral density and an increased risk of reduced bone density (osteoporosis) or bone fracture because a lack of vitamin D alters mineral metabolism in the body.

The VDR may be involved in cell proliferation and differentiation. Vitamin D also affects the immune system, and VDRs are expressed in several white blood cells, including monocytes and activated T and B cells (Watkins *et al.,* 2015). Apart from VDR activation, various alternative mechanisms of action are under study, such as inhibition of signal transduction by hedgehog, a hormone involved in morphogenesis (Sarkar *et al.,* 2010).

Vitamin K in hunam is involved in the carboxylation of certain glutamate residues in proteins to form gamma-carboxyglutamate (Gla) residues. The modified residues are often (but not always) situated within specific protein domains called Gla domains. Gla residues are usually involved in binding calcium, and are essential for the biological activity of all known Gla proteins (Furie *et al.,* 1999) At this time, 17 human proteins with Gla domains have been discovered, and they play key roles in the regulation of three physiological processes: such as Blood coagulation: prothrombin (factor II), factors VII, IX, and X, and proteins C, S, and Z (Mann*,* 1999), Bone

metabolism: osteocalcin, also called bone Gla protein (BGP), matrix Gla protein (MGP) (Price, 1988) periostin (Coutu *et al.,* 2008) and the recently discovered Gla-rich protein (GRP) (Viegas *et al.,* 2008; Viegas *et al.,* 2009), Vascular biology: growth arrest-specific protein 6 (Gas6) (Hafizi *et al.,* 2006). Like other lipid-soluble vitamins (A, D and E), vitamin K is stored in the fatty tissue of the human body.

The function of vitamin K_2 is to add carboxylic acid functional group to a glutamate (Glu) amino acid residue in a protein, to form a gamma-carboxyglutamate (Gla) residue. This is a somewhat uncommon posttranslational modification of the protein, which is then known as a "Gla protein". The presence of two −COOH (carboxylic acid) groups on the same carbon in the gamma-carboxyglutamate residue allows it to chelate calcium ions. The binding of calcium ions in this way very often triggers the function or binding of Gla-protein enzymes, such as the so-called vitamin K-dependent clotting factors discussed below.

Within the cell, vitamin K undergoes electron reduction to a reduced form called vitamin K hydroquinone, catalyzed by the enzyme vitamin K epoxide reductase (VKOR) (Oldenburg *et al.,* 2006). Another enzyme then oxidizes vitamin K hydroquinone to allow carboxylation of Glu to Gla; this enzyme is called gamma-glutamyl carboxylase (Suttie, 1985; Presnell *et al.,* 2002) or the vitamin K-dependent carboxylase. The carboxylation reaction only proceeds if the carboxylase enzyme is able to oxidize vitamin K hydroquinone to vitamin K epoxide at the same time. The carboxylation and epoxidation reactions are said to be coupled. Vitamin K epoxide is then reconverted to vitamin K by VKOR. The reduction and subsequent reoxidation of vitamin K coupled with carboxylation of Glu is called the vitamin K cycle (Stafford, 2005).

Warfarin and other 4-hydroxycoumarins block the action of VKOR (Whitlon *et al.,* 1978). This results in decreased concentrations of vitamin K and vitamin K hydroquinone in tissues, such

that the carboxylation reaction catalyzed by the glutamyl carboxylase is inefficient. This results in the production of clotting factors with inadequate Gla. Without Gla on the amino termini of these factors, they no longer bind stably to the blood vessel endothelium and cannot activate clotting to allow formation of a clot during tissue injury. As it is impossible to predict what dose of warfarin will give the desired degree of clotting suppression, warfarin treatment must be carefully monitored to avoid overdose.

All the parameter compared in table 4.8 were all found to be statistically significantly different at 0.05 level except age which shows a very high significant difference ($P > 0.116$) between osteoarthritis and Non osteoarthritis subjects. Expectedly, this study further revealed that functional disability was more prevalent amongst respondents living below poverty level and those who lack formal education. The same trend has been observed in the studies reviewed (Mont, 2007; W.H.O, 2014). Poverty, ignorance, diseases and consequent disability is a vicious cycle, which Mont asserted are inseparable (Mont, 2007). Poverty leads to malnutrition, poor health services and sanitation, as well as unsafe living and working conditions, which are all associated with disability. This may reflect a reporting bias: poor access to medical service as well as a high level of illiteracy, which would limit the number of elderly persons who might be aware of having a medical condition (Gureje *et al*., 2006; Goldman *et al.,* 2003). Some of the morbidities were associated with a higher prevalence of functional disability, although this was not statistically significant. These include cataract, osteoarthritis, diabetes mellitus, glaucoma, UTI and RTI. Cataract and glaucoma are the major causes of visual impairment, which will obviously affect the ability to carry out BADL. Osteoarthritis reduces joint mobility and inflicts pain, which may explain why its occurrence in this study was associated with a higher prevalence of functional disability.

Furthermore, there are conflicting reports as to whether vitamin D supplementation decreases pain in OA (Rachel *et al*., 2017). In a 2-year randomized controlled study in which symptomatic knee OA patients were given oral doses of cholecaliciferol to raise serum levels of vitamin D, there was no reduction in Western knee pain scores (McAlindon *et al*., 2013). In a double-blind study with 103 knee OA patients, patients receiving vitamin D oral supplements had slightly less pain compared with those receiving placebos after 1-year follow-up (Sanghi *et al*., 2013); however, these patients were not as physically capable as their placebo counterparts.

Despite a plausible biological rationale and a positive observational study (Neogi *et al*., 2006), many findings do not support a significant effect of vitamin K supplementation on osteoarthritis for all persons. Of note, despite previous studies demonstrating an association between poor vitamin K status and bone health (Cockayne *et al*., 2006), the parent trial did not demonstrate a significant effect of vitamin K supplementation on bone mineral density in the hip or spine (Booth *et al*., 2008).

It is possible that modest daily vitamin D supplementation was potentially beneficial in this study and thus may play a potential role that will benefit patients with ostheoarthritis.

5.2 Conclusion

In conclusion, result shows patients with osteoarthritis had higher levels of Cu and lower levels of serum Zn and Se. in addition, Zinc and selenium supplements which reduces the severity of OA should be further encuraged and investigated. vitamin D supplementation in amounts that are achievable in the diet, when taken with recommended amounts of calcium does confer additional benefit and may contribute greatly in releiving the effects of joint related symptoms in

osteoarthritis, particularly in older adults who were not necessarily insufficient in vitamin D. Therefore, modest daily vitamin D supplementation was potentially beneficial in this study and thus may play a potential role that will benefit patients with ostheoarthritis.

Treatment of vitamin K deficiency, which is common in older people, may have yet unproven favourable effect on osteoarthritis in aliviating the pains.

5.3 Recommendation

It is highly recommended that all the serum samples of elderly subjects diagnosed of osteoathritis be tested for basic structural trace elecment routinely as part of clinical diagnosis. Analysis of structural trace element should be encouraged as normal management style in ostheoathritis patients this will bring save management of subject with ostheoathritis. Introduction of major vitamins that help stabilis pain and nurishment of the body should be encouraged for use in all centers managing patients with Ostheoathritis. Research efforts aimed at the discovery of anti athritis agents that target the artherosmuscular organs should also be encouraged.

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