EVALUATION OF BIOMARKERS OF CALCIUM REGULATION AND OXIDATIVE STRESS AMONG PREGNANT AND LACTATING WOMEN IN ZARIA, NIGERIA

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BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN HUMAN PHYSIOLOGY

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ZARIA

DECEMBER, 2017

Declaration

I declare that the work in this thesis "EVALUATION OF BIOMARKERS OF CALCIUM REGULATION AND OXIDATIVE STRESS AMONG PREGNANT AND LACTATING WOMEN IN ZARIA, NIGERIA" has been carried out by me in the Department of Human Physiology under the supervision of Prof. A. J. Randawa, Dr. M. U. Kawu and Prof. A. Mohammed. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any other institution.

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Certification

This thesis entitled "EVALUATION OF BIOMARKERS OF CALCIUM REGULATION AND OXIDATIVE STRESS AMONG PREGNANT AND LACTATING WOMEN IN ZARIA, NIGERIA" by Ohunene Makoju Avidime meets the regulations governing the award of Doctor of Philosophy degree in Human Physiology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This work is dedicated to Almighty God, who makes everything beautiful in His time and to my mum, Mrs. Bilkisu Caroline Makoju. May God rest your gentle soul, amen.

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Abstract

Pregnancy and lactation are normal but stressful physiological phenomena during which many biochemical changes occur, including calcium and phosphate metabolism, which may lead to calcium deficiency in pregnant women. Also the oxidative stress (ODS) associated with pregnancy and lactation induces increased activity of biomarkers of ODS. Since data on the serum levels of calcium, phosphate and ODS biomarkers in women in Zaria are scanty, the evaluation of their serum profiles in pregnant and lactating women in Zaria will provide reference data for clinicians and other medical workers involved in routine antenatal care in Nigeria and other resource limited third world countries. The aim of this study was to evaluate the changes in serum levels of calcium, phosphate, vitamin D and their regulatory hormones and serum antioxidant status of pregnant women in Zaria, Nigeria. In this study, blood samples were collected and anthropometric measurements such as weight, height and body mass index (BMI) of 179 women were taken. The women were grouped into: non-pregnant women: control group (n = 45), pregnant women in the first trimester (n = 45), pregnant women in the second trimester (n = 45), pregnant women in the third trimester (n = 45) and lactating women (n = 30). Serum was obtained from the blood samples and assayed for calcium, phosphate, vitamin D, parathyroid hormone, calcitonin, antioxidant biomarkers (superoxide dismutase, catalase and glutathione peroxidase) and lipid peroxidation biomarker (malondialdehyde) using standard methods. Data were presented as mean ± SD. Analysis of data was performed using one-way analysis of variance (ANOVA) to find the significance between the groups, followed by Holm-Sidak post-hoc test. Pearson's correlation analysis was used to determine the inter-relationship between the variables and values of $p \leq 0.05$ were considered significant. Statistical package used was Minitab statistical software version 16. The results revealed that there was a significant decrease in serum calcium concentration ($p < 0.01$) during the third trimester of pregnancy and during lactation. There was increased serum concentration of vitamin D, parathyroid hormone and calcitonin in the 2^{nd} trimester which were decreased during the third trimester and lactation, these findings were not significant when they were compared with the control. Only 1.76% of hypocalcaemia was observed in the study population and 21.24% of hypervitaminosis D was observed. There was a negative correlation observed between serum calcium concentration and gestational age ($r = 0.255$) which was significant ($p < 0.01$). There was no correlation observed between gestational age and serum phosphate concentration. Lipid peroxidation was highest in the lactating women since serum malondialdehyde concentration was highest here, and it was significant when compared with the control. Serum superoxide dismutase and glutathione peroxidase concentrations were significantly higher during lactation when compared with the control ($p < 0.05$). There was significant negative correlation ($r = -0.580$, $p = 0.001$) between serum malondialdehyde concentration and serum glutathione peroxidase concentration. There was also a significant positive correlation between serum superoxide

dismutase and vitamin D concentrations ($r = 0.175$, $p < 0.05$), serum glutatathione peroxidase and vitamin D concentrations ($r = 0.227$, $p < 0.01$) and serum malondialdehyde and phosphate concentrations ($r = -0.189$, $p = 0.05$). There was no significant difference observed in the mean serum concentrations of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin between the age groups. There was also no significant difference in the mean serum concentrations of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin on parity when compared with the control. No significant difference was also observed when serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin were compared among the ethnic groups although serum calcitonin concentration appeared highest among the Hausa ethnic group, while serum concentration of catalase was highest among the Yorubas. Serum calcium concentrations appeared to be highest among women who did handwork, though not significant. Serum concentration of malondealdehyde was highest in house wives though not significant. BMI and residential location did not seem to affect serum concentrations of all the parameters. Based on the findings of this study, it was concluded that there was very low prevalence of serum calcium, phosphate and vitamin D deficiency in pregnant and lactating women in Zaria; even though the serum concentration of calcium decreased across the trimesters and lactation, malondialdehyde concentration increased during lactation, while superoxide dismutase activity and reduced glutathione concentration were significantly higher. Local base-line reference values for calcium, phosphate, vitamin D, parathyroid hormone and calcitonin for pregnant women and a predictive equation for the serum calcium levels during pregnancy and the period of lactation were established in the study.

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Abbreviations

1,25(OH)2D	-	1,25-dihydroxyvitamin D
25(OH)D	-	25-hydroxyvitamin D
7DHC	-	7-Dehydrocholesterol
ADH	-	Autosomal Dominant Hypocalcaemia
ANOVA	-	Analysis of variance
BMC	-	Bone mineral content
BMD	-	Bone mineral density
BMI	-	Body mass index
cAMP	-	Cyclic adenosine monophosphate
CaSR	-	Calcium sensing receptor
CAT	-	Catalase
CGRP	-	Calcitonin gene-related peptide
CKD	-	Chronic kidney disease
CT	-	Calcitonin
CTR	-	Calcitonin receptor
CTRKO	-	Calcitonin receptor knockout
CTX	-	Cross-linked C-telopeptide
DBP	-	Vitamin D binding protein
DRIs	-	Dietary Reference Intakes
DXA	-	Dual X-ray absorptiometry
EAR	-	Estimated average requirement
EAR	-	Estimated Average Requirement
ELISA	-	Enzyme-linked immunoassay
FAO	-	Food And Agriculture Organization

FGF23	-	Fibroblast growth factor 23
FHH	-	Familial Hypocalciuric Hypercalcaemia
GDM	-	Gestational Diabetes Mellitus
GFR	-	Glomerular filteration rate
GnRH	-	Gonadotropin releasing hormone
GPCRs	-	G protein-coupled cell surface receptors
GPx	-	Glutathione peroxidase
GSH	-	Glutathione
GSH-Rx	-	Glutathione reductase
IRMA	-	Immunoradiometric assay
IRMAs	-	Immunoradiometric assays
IU	-	International Unit
IU/d	-	International Units per day
LRP5	-	Lipoprotein receptor-related protein 5
MAVIDOS	-	Maternal Vitamin D Osteoporosis Study
MDA	-	Malondialdehyde
mg/d	-	milligrams per day
mg/dL	-	milligrams/deciliter
mg/kg	-	milligrams per kilogram
mmol/L	-	millimoles/litre
ng/L	-	Nanograms/Litre
NHANES	-	National Health and Nutrition Examination Survey
nM	-	Newton metre
NSHPT	-	Neonatal Severe Hyperparathyroidism
pg/mL	-	Picograms per millilitres

PTH	-	Parathyroid hormone
PTHrP	-	Parathyroid hormone-related protein
PTHRs	-	Parathyroid hormone receptors
QCT	-	Quantitative computed tomography
RDA	-	Recommended Dietary Allowance
ROS	-	Reactive Oxygen Species
SNPs	-	Single nucleotide polymorphisms
SOD	-	Superoxide dismutase
TAC	-	Total Antioxidant Capacity
TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric acid reactive substances
TCA	-	Trichloroacetic acid
TRPV6	-	Transient receptor potential cation channel subfamily V
VDR	-	Vitamin D receptor
WHO	-	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

It has been well established that calcium plays an essential role in both the development and maintenance of bone health. Although osteoporosis is a condition of childhood that presents in older age, the importance of calcium homeostasis and optimum calcium intake throughout the lifespan is crucial in the prevention of senile osteoporosis (Hacker-Thompson *et al.*, 2012).

There is evidence suggesting that maternal calcium intake can affect the bone development of the foetus and its future skeletal growth. Thus, it is essential that women of child-bearing age are educated on the importance of meeting their calcium requirements. Women of child-bearing age will meet their own needs and those of their infants, if they regularly consume adequate amounts of calcium (1,000 mg/day). Additional calcium supplementation during pregnancy appears to have the greatest impact in women who chronically consume <500 mg calcium/day, demonstrating the importance of adequate calcium intake before pregnancy begins (Hacker-Thompson *et al.*, 2012).

Calcium and phosphate play many essential roles in the body. To accomplish these functions, serum levels need to be maintained in a certain range with a complex interplay of hormones involved to maintain a balance (Fukumoto, 2014). The most important factors involved in the balance are vitamin D and parathyroid hormone. Vitamin D regulates absorption of calcium from the gastrointestinal tract as well as its excretion from kidney. While, serum phosphate level is regulated by intestinal

phosphate absorption, renal phosphate handling and equilibrium of extracellular phosphate with that in bone or intracellular fluid. Several hormones such as parathyroid hormone mainly functions through conversion of vitamin D to its active metabolite (1,25-hydroxycholecalcipherol) in the kidneys, which regulates intestinal absorption, renal excretion, and exchange between the extracellular fluid and bone (Levine *et al*., 2014). Calcitonin is a hormone secreted from thyroid and is involved in calcium homeostasis, but far less important than parathyroid hormone (PTH). It primarily has calcium-lowering effect (Kumar, 2009). Calcium homeostasis is a function of dietary intake, intestinal absorption, skeletal accretion and resorption and urinary excretion.

The key organs involved in the changes in calcium metabolism during pregnancy include the maternal parathyroid gland, intestine, and the foetoplacental unit (Kovacs, 2008). Pregnancy and lactation cause a substantial increase in demand for calcium that is met by different maternal adaptations within these periods. This adaptation essentially involves increased intestinal calcium absorption, which more than doubles during pregnancy and maternal bone resorption to provide most of the calcium content of breast milk during lactation (Kovacs, 2008). The pregnant woman's body provides 25 to 30 g of calcium to support the developing foetal skeleton (Holt, 2013). Much of the foetal calcium demand occurs in the third trimester of pregnancy, when the foetal skeleton undergoes mineralisation (Holick *et al*., 2012).

Vitamin D deficiency has long been associated with poor bone development and has been identified as the cause of rickets. Although the incidence of rickets has declined with the current daily recommendations of vitamin D intake, the prevalence and additional consequences of low serum vitamin D levels have not been recognized until

recently (Wagner and Greer, 2008; Holick *et al*., 2012). Measurement of serum vitamin D in pregnancy has helped researchers to establish the prevalence of vitamin D deficiency and elucidate adverse maternal and foetal outcomes associated with it (Bodnar, 2007). Prevention of these diseases and reduction of the risk for childhood illnesses that are linked to early vitamin D deficiency are possible with greater understanding of vitamin D physiologic components, risk factors for vitamin D deficiency and methods of supplementation to attain optimal levels in pregnant and lactating women (Mulligan *et al.,* 2010).

Vitamin D is a prohormone that is derived from cholesterol. The nutritional forms of vitamin D include D_3 (cholecalciferol), which is generated in the skin of humans and animals and vitamin D_2 (ergocalciferol), derived from plants. Both forms are absorbed in the gut and used by humans (Mulligan *et al.,* 2010). Vitamin D occurs naturally in fish and some plants, but is not found in significant amounts in meat, poultry, dairy products (without fortification) or the most commonly eaten fruits and vegetables. Recommended Dietary Allowances (RDAs) for vitamin D is between 600 to 800 IU/d for both pregnant and non-pregnant individuals aged 0-50 years, while the RDA for calcium ranges from 700 to 1300 mg/d (Ross *et al*., 2011).

In Nigeria, high calcium foods include groundnut cake (*kulikuli*) (871.08 mg/100 g), beans cake (*akara*) (296.13mg/100g) and cassava meal (*fufu*) (255.31mg/100g) (Agbon *et al.,* 2010). Other foods include milk, bones, cartilage and tofu (*wara*). Wild salmon (Titus) (99.223 g) provides 600-1000 IU; farmed salmon has approximately 25% of this amount per serving (Lu *et al.,* 2007). The same amount of mackerel, sardines or tuna (*kote*) fish provides 200-300 IU. Cod liver oil (1 tsp) provides 600-1000 IU. In the

United States, the major dietary sources of vitamin D are fortified foods (Mulligan *et al.*, 2010). For example, 226.8 g of fortified milk, orange juice or yoghurt, 85.1 g of fortified cheese or a serving of fortified breakfast cereal each provides 100 IU of vitamin D (Ko *et al*., 2008). Increased plasma PTH maintains serum calcium in the reference range by enhancing renal production of $1,25(OH)_2D$, increasing bone turnover, accelerating bone loss and promoting tubular calcium reabsorption and phosphate excretion. Increased $1,25(OH)_2D$ induces intestinal calcium and phosphorus absorption and stimulates osteoclastic activity, thereby increasing calcium and phosphorous availability in the blood (Dusso *et al*., 2005).

Reports show that normal pregnancy is accompanied by a high metabolic demand and elevated requirements for tissue oxygen which results in increased oxidative stress and antioxidant defences (Idonije *et al*., 2011; Ogbodo *et al*., 2014). Oxidative stress is the presence of reactive oxygen species (ROS) in excess of the buffering capacity of available antioxidants (Palan *et al*., 2001). Although the generation of ROS is a normal physiological process, their increased production in the body causes lipid peroxidation (Tiwari *et al*., 2010). It has been suggested that ROS are likely promoters of maternal vascular malfunction, because ROS particularly superoxide anions evoke endothelial cell activation (Ogbodo *et al*., 2014). The ROS have been implicated in atherosclerosis, cancers, pre-eclampsia and many other human diseases (Zhang *et al*., 2001). The antioxidant defence mechanisms in the body are categorized into two types - ROS scavenging and chain-breaking antioxidants. The ROS scavenging mechanisms include enzymatic antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GSH-Rx) and catalase, which limit the cellular concentration of ROS and prevent excessive oxidative damage (Scott *et al.*, 1994).

The malondialdehyde (MDA) concentration gradually increases in pregnant women from the 1st to 3rd trimester, while antioxidants, SOD, GPx, GSH-Rx and catalase were found to be lowest in the 3rd trimester of pregnancy (Patil *et al.,* 2007). In a study carried out to investigate the effects of calcium and vitamin D supplementation on the metabolic status of pregnant women with gestational diabetes mellitus (GDM), findings showed decreased plasma MDA, but no change in plasma total antioxidant capacity (TAC) (Asemi *et al*., 2014).

During pregnancy and lactation, significant changes in maternal vitamin D and calcium metabolism occur to provide the calcium that is needed for foetal bone mineral deposition. During the first trimester, the foetus accumulates 2-3 mg/day in the skeleton; however, this rate of accumulation doubles in the last trimester (Abrams, 2007). Similarly, decreased levels of $25(OH)_2D$ has been reported in pregnant women (Cho *et al*., 2013). The body of a pregnant woman adapts to foetal requirements by increasing calcium absorption in early pregnancy, with maximal absorption in the last trimester (Cross *et al.,* 1995; Ritchie *et al.,* 1998).

The profound increase in intestinal calcium absorption cannot be explained solely by the increased $1,25(OH)_2D$, because the increased calcium absorption occurs before $1,25(OH)_2D$ levels increase. In rodents, the increase occurs even in the absence of vitamin D receptor (Kovacs, 2011). Other signals that regulate active calcium homeostasis and vitamin D synthesis during pregnancy include prolactin, placental lactogen, calcitonin, osteoprotegerin and estrogen. Prolactin increases throughout pregnancy and remains above normal after delivery. Calcitonin increases two-fold in the second trimester, compared with the first trimester, but then declines slightly at

term. It has been hypothesized that the rise in serum calcitonin protects the maternal skeleton from excessive resorption of calcium from the bones (Mulligan *et al.*, 2010).

Maternal nutrition during pregnancy is known to have an effect on foetal growth and development. It is recommended that women increase their calcium intake during pregnancy and lactation, although the recommended dosage varies among professionals (from paediatricians to nutritonists) (Buppasiri *et al.*, 2011). At birth, an infant's body contains 20–30 g calcium, almost all of which is in the skeleton (FAO/WHO, 2004; Pettifor, 2008). In a study by Benali and Demmouche (2014) in Algeria, the prevalence of hypocalcaemia in pregnant women was 70.55%. Sanchez *et al.* (1997) reported 10 to 15% decrease in calcium levels from the first to the third trimester in 30 pregnant teenagers in Maiduguri, Northeast Nigeria.

Vitamin D deficiency during pregnancy is a world-wide epidemic. Studies have reported a prevalence that ranges from 18-84%, depending on the country of residence and local clothing customs (Sachan *et al*, 2005; Van der Meer *et al.*, 2006; Dawodu and Wagner *et al*., 2007). In the United States, vitamin D deficiency is estimated to occur in 5-50% of pregnant women (Lee, 2007). African American women have a much higher risk of vitamin D deficiency, compared with other women because of increased skin pigmentation and low dietary intake (Nesby-O'Dell, 2002). Bodnar *et al*., (2007) reported the prevalence of vitamin D deficiency and insufficiency in 200 white and 200 black pregnant women and in the cord blood of their neonates. At delivery, vitamin D deficiency and insufficiency occurred in 45.6 and 46.8% of black neonates, respectively, compared with 9.7 and 56.4% of white neonates (Mulligan *et al.*, 2010). In a study by Lee *et al*. (2007), it was reported that although the majority of

mothers received daily prenatal multivitamin supplementation, vitamin D deficiency was found in 50% of pregnant women. Similarly, Wang *et al.* (2010) reported a large proportion of women (57.1%) with vitamin D deficiency in Chengdu, China.

The erythrocyte membrane is involved in stability of the membrane and other functions due to the presence of fatty acids, phospholipids and cholesterol (Jimoh, *et al*., 2015). In experiments, erythrocytes are used to evaluate increased production of ROS as a result of oxidative stress which occurs due to the high levels of poly-unsaturated fatty acids content of the erythrocyte membrane and continuous exposure of haemoglobin to increased levels of oxygen and iron. This makes the erythrocyte a useful tool in study models for oxidative stress (Yaqub *et al*., 2014). Increased generation of ROS occurs during oxidative stress, resulting in osteoporosis and osteomalacia which impair calcium homeostasis. The impaired calcium homeostasis affects the control of calcium and its associated hormones in the body (Yadav *et al.*, 2014).

1.2 Statement of Research Problem

Pregnancy is a normal but stressful physiological phenomenon during which many biochemical changes occur, including deranged calcium metabolism, which may lead to calcium deficiency in pregnant women (Benali and Demmouche, 2014). It is accompanied by high-energy demand and an increased oxygen requirement. Augmented levels of oxidative stress may occur because of the increased cellular uptake and utilisation of oxygen, associated with pregnancy (Idonijie, 2011). It has been observed that pregnant women are more susceptible to oxidative damage than non-pregnant women, as evidenced by decreased antioxidants in pregnant women (Patil *et al.*, 2007; Ugwa *et al*., 2014). The results of biochemical tests including

calcium-related factors and oxidative stress parameters during pregnancy may therefore, differ from the normal reference ranges. The values of the parameters may be wrongly interpreted as abnormal, leading to unnecessary and potentially dangerous therapeutic interventions. Towards the end of gestation, there appears to be calcium deficiency and decreased levels of parathyroid hormone in pregnant women (Sanchez *et al*., 1997; Ainy *et al*. 2006). Pregnancy and lactation cause a substantial increase in demand for calcium that is met by different maternal adaptations within each period. Therefore, measures taken towards providing adequate diet and supplementation of calcium and inorganic phosphate during this period may be of value in preventing deleterious effects due to the deficiency or reduced levels in both mother and the foetus (Ikekpeazu *et al*., 2010).

1.3 Justification of the Study

Calcium deficiency is common in pregnant women (Benali and Demmouche, 2014). Despite the importance of normal calcium and vitamin D metabolism during pregnancy and lactation, available data on the prevalence of vitamin D and calcium deficiency during pregnancy in Nigerian women is scarce. During pregnancy, there is a variation in the levels of serum calcium and inorganic phosphate at different stages of pregnancy**.** Therefore, by measuring the levels of calcium, inorganic phosphate, vitamin D and their regulatory hormones at various stages of pregnancy, local reference values may be established that will contribute to the growing body of knowledge concerning calcium and phosphorus disorders during pregnancy in Nigerian women. Furthermore, evaluating the activity of serum biomarkers of oxidative stress may provide useful information on the role of oxidative stress in the occurrence of

calcium and vitamin D disorders in pregnant women. Such studies are lacking in the available literature.

The result of this study may estabilish the relationship between calcium and its hormonal control mechanisms in pregnant women in Zaria, and its environs which has not been investigated. Overall, the results of this investigation could be beneficial to the improvement of the management of pregnant women presenting with calcium associated disorders.

1.4 Aim

The study is aimed at evaluating the changes in serum levels of calcium, phosphate, vitamin D and their regulatory hormones and serum antioxidant status of pregnant women in Zaria, Nigeria.

1.5 Specific Objectives

1. To determine the prevalence of calcium, phosphate and vitamin D deficiency in pregnant women in Zaria.
2. To determine baseline data on serum concentrations of calcium, inorganic phosphate, 25-hydroxyvitamin D, parathyroid hormone (PTH) and calcitonin in pregnant women in Zaria.
3. To formulate a predictive equation for serum calcium levels during the different trimesters of pregnancy and the period of lactation.
4. To assess biomarkers of oxidative stress: malondialdehyde concentration, activities of superoxide dismutase, catalase and glutathione peroxidase in pregnant women in Zaria.

5. To determine the relationship between serum concentrations of calcium, phosphate and vitamin D and the biomarkers of oxidative stress in pregnant women in Zaria.
6. To determine the influence of age, different trimesters of pregnancy and body mass index (BMI) on serum concentration of calcium, phosphate, 25-hydroxyvitamin D, PTH and calcitonin.
7. To determine the effect of ethnicity, occupation and residential location on serum concentrations of calcium, phosphate, vitamin D, calcitonin and biomarkers of oxidative stress.

1.6 Research Hypothesis

Pregnancy, lactation and socio-cultural factors do not affect serum levels of calcium, phosphorus, vitamin D, parathyroid hormone and calcitonin and the antioxidant enzymes status of pregnant women in Zaria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Calcium Metabolism during Pregnancy

Pregnancy is a dynamic, anabolic state. Within several weeks of conception, a new endocrine organ, the placenta, is already formed and is secreting hormones that affect the metabolism of all nutrients. These adjustments in nutrient metabolism, in addition to changes in the anatomy and physiology of the mother, support foetal growth and development, while maintaining maternal homeostasis and preparing for lactation. Depending on the nutrient, one or more of the following adjustments occur: 1) accretion in new tissue or deposition in maternal stores, 2) redistribution among tissues, and 3) increased turnover or rate of metabolism. To support these adjustments, the use of nutrients from the diet may be altered either by increasing intestinal absorption or by reducing excretion via the kidney or gastrointestinal tract (Fukumoto and Shimizu, 2011).

These adjustments in nutrient metabolism are complex and evolve continuously throughout pregnancy. The changes in nutrient metabolism can be described by several general concepts: adjustments in nutrient metabolism are driven by hormonal changes, foetal demands, and maternal nutrient supply; more than one potential adjustment exists for each nutrient; maternal behavioural changes augment physiologic adjustments; and a limit exists in the physiologic capacity to adjust nutrient metabolism to meet pregnancy needs, which when exceeded, foetal growth and development are impaired. Some of the parameters involved are calcium and phosphate levels together with the calciotropic hormones such as parathyroid hormone, calcitonin, vitamin D and fibroblast growth factor 23 (Fukumoto and Shimizu, 2011).

2.2 Calcium and Phosphate

Calcium is found in the body principally in calcium phosphate form in bones. Surplus amounts of calcium ion in extracellular fluid can cause the heart to stop in systole. At the other extreme, decreased levels of calcium can trigger impulsive release of signals from nerve fibers, bringing about tetany (Guyton and Hall, 2011). Phosphate is the main anion of intracellular fluid, it has the capacity to combine reversibly with several coenzyme systems and with numerous other compounds that are essential for the processes of metabolsm. In serum, phosphorus is mainly found as inorganic phosphates (dihydrogen and monohydrogen phosphate), while in bone it is principally in the form of hydroxyapatite, and in soft tissues and extracellular fluid there is an abundance of organic phosphates complexed with carbohydrates, lipids, and proteins (Kovacs, 2016). Several vital reactions of phosphates such as those associated with bone contain a great quantity of calcium phosphate (Guyton and Hall, 2011).

2.2.1 Sources of calcium

In Nigeria, high calcium foods include groundnut cake (*kulikuli*) (871.08 mg/100 g), beans cake (akara) (296.13mg/100g) and cassava meal (*fufu*) (255.31mg/100g) (Agbon *et al*., 2010). Other calcium rich foods include milk, bones, cartilage and tofu (*wara*).

2.2.2 Normal blood calcium levels

The corrected total serum calcium concentration is normally 8.5-10.2 mg/dL, but there is no sure means of predicting the serum calcium level, for either hypocalcaemia or hypercalcaemia, at which symptoms will occur. The rapidity of change, as well as the absolute serum calcium concentration, impacts symptom development. The amount of calcium in the blood changes as we grow older. The highest serum calcium levels are

seen in teenagers and young adults. By the age thirty five to forty years, the blood calcium decreases to less than 10 mg/dL majority of the time.

The reference range for serum calcium used at the Chemical Pathology Laboratory, Ahmadu Bello University Teaching Hospital, Shika, Zaria is 2.25 – 2.75 mmol/L, while the reference range for phosphate level is 0.8 – 1.4 mmol/L in adults and 0.8 – 1.9 mmol/L in children. There are no reference ranges for pregnant women.

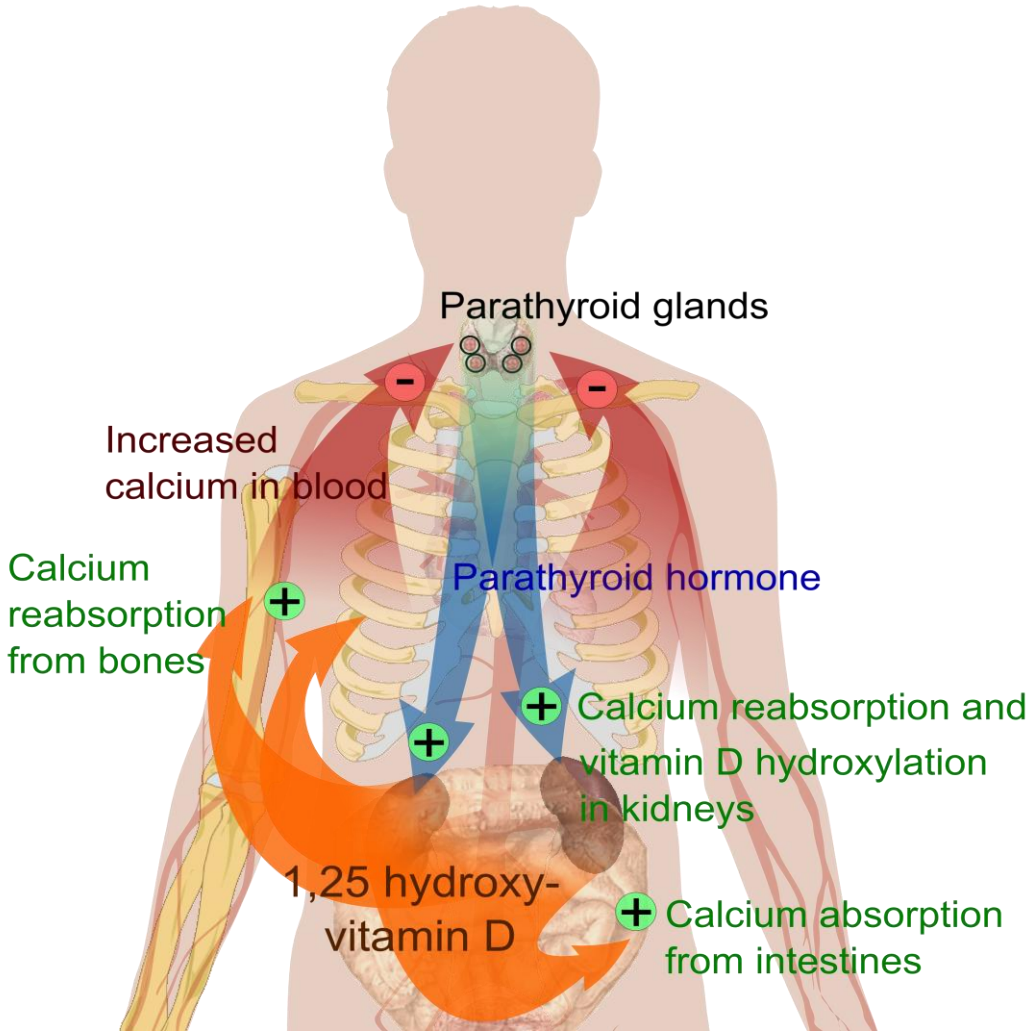


Figure 2.1: Calcium regulation (Boundless Biology, 2017)

During pregnancy and lactation, the female physiology must adapt to meet the added nutritional demands of the foetus and neonate. It must determine exactly how: 1) the demand for the transfer of calcium and other minerals will be met; 2) The specific adaptations resorted to during pregnancy and lactation that will sustain these demands (the interplay of hormones); 3) how these adaptations modify the natural biochemical and hormonal constituents of mineral and skeletal metabolism; 4) What effect these adaptations have during pregnancy and lactation on preexisting maladies of mineral and bone metabolism. Even with some of these adjustments, there are still long-term beneficial or adverse consequences of pregnancy and lactation on the skeletal health of women (Kovacs, 2016).

The foetal demand for minerals which the pregnant woman must supply, has been established by evaluating the ash weight and mineral content of foetal cadavers between 24 weeks and term. Multiple studies have demonstrated that the average full-term foetus has ~ 30 g calcium, 20 g phosphorus, and 0.80 g magnesium (Kovacs, 2016). Though, foetal mineral content is not found at a constant proportion in pregnancy; in its place, not less than 80% of the calcium, phosphorus, and magnesium present in a human term foetus is accreted by 28 - 30 weeks equivalent to the third trimester. This is consistent with a mean calcium transfer rate of 100–150 mg/kg per day which, for a normal foetus, is ~ 60 mg/day at week 24 and 300–350 mg/day towards the end of the third trimester.

In the same way, the rate of phosphorus transport is ~ 40 mg/day at week 24 and increases to 200 mg/day over the last 5 weeks of gestation. The rate of magnesium transfer increases more modestly from 1.8 to 5.0 –7.5 mg/day over the last 5 weeks

(Kovacs, 2016). When represented as hourly values, during the third trimester the foetal demand for calcium and phosphorus represent between 5 and 10% of the quantity found in the mother's plasma. This means that the foetal demand for calcium and phosphorus has the potential to provoke maternal hypocalcaemia and hypophosphatemia. The mineral requirement for neonate, which may be provided during breastfeeding, has mostly been deduced from experiments where well-nourished neonates are weighed before and after each feed; a few investigations have measured alteration in the mother's weight or the quantity of milk that can be expressed by hand. The calcium consumption has been the main parameter measured in these calculations.

These studies revealed an average consumption of 780 ml of human milk per day, which, combined with an average calcium content in milk of 260 mg/L in the first 6 months postpartum (Jensen, 1995), shows a total calcium intake of ~ 200 mg/day. Estimates of fractional calcium absorption from metabolic balance studies have shown that neonates absorb ~60–70% of calcium from human milk (Abrams, 2010; Hicks *et al.*, 2012), thereby making ~120–140 mg of calcium available for the skeleton to potentially take up each day. Fractional calcium absorption is proportional to intake and facilitated by lactose in neonates and infants, and can be as low as 30–40% when formula (which has twice the calcium content as human milk) is consumed (Abrams, 2010).

Estimates of neonatal mineral accretion are another indication of how much mineral is truly required, but the results have been much more variable due to different techniques, variable body sizes, ethnicity, cross-sectional versus longitudinal studies, small sample sizes, and other factors. For example, direct measurements of ash weight

and mineral content have been made in cross-sectional studies of cadaveric specimens, whereas indirect measurements of mineral content (such as through radiological techniques) have been made *in vivo* in longitudinal studies of healthy neonates and infants. One widely quoted study lacked any neonatal cadavers, but extrapolated from the mineral content of newborn, childhood, and adult cadavers to infer that the calcium content (the most abundant mineral in bone) increased 50 g by the end of the first year, which corresponds to a daily accretion rate of ~ 140 mg (Leitch and Aitken, 1959). On the other hand, other evaluations of cadaveric specimens have proposed smaller accretion rates of 30–50 mg/day in the first 6 months.

When mineral matter has been deduced from peripheral radiographs or by dual X-ray absorptiometry (DXA), the day by day accretion rates have alternated from 80 mg to an idealistic 380 mg; the second study denotes problems in calibrating DXA against true mineral content of neonates and infants. An overall consensus is that normal milk output, and its normal calcium content, are more practical indicators of the average neonatal prerequisite for calcium (Institute of Medicine, 2011).

These calculations were done by the Institute of Medicine to establish the estimated average requirement (EAR) for calcium consumption, and deduced that breastfeeding needs that an average of 200 mg calcium be delivered daily through milk to a singleton during the first 6 months. From this intake the neonatal skeleton is expected to accrete ~ 100 mg of calcium daily (Institute of Medicine, 2011). Though, the output of milk on an individual basis is influenced by the suckling demands of the neonate and can definitely distinctly exceed these ranges. Those women with twins and triplets that

suckle their babies can have, respectively, more than double and triple, respectively, the milk output of women who nurse singletons (Dardenne *et al.*, 2003).

Some specific cases of women nursing singletons have been reported with milk output of upwards of 2.4 –3.1 litres per day that was maintained for more than 12 months of lactation, the composition of milk is similar between women with average and high outputs (Kovacs, 2016), and so increased milk production will trigger increased maternal calcium loss. During the period of 6 to 12 months of age, the diet of the infant begins to incorporates solid food even with continued breastfeeding.

There are far less studies that have studied this period of infancy and so there is paucity of information about it. The average calcium content of human milk is fairly low at 200 mg/l, and the intake is less at ~ 600 ml/day, which implies that the infant has an approximated calcium intake of 120 mg/day from human milk. A supplementary 140 mg/day of calcium is projected to come from solid foods making the total calcium intake by the infant to be 260 mg/day (Institute of Medicine, 2011). In earlier cadaveric reports, there was a continued daily accretion rate of ~ 140 mg during the 7th to 12th months; however another report concluded that calcium accretion is ~ 50 mg/day during this time (Institute of Medicine, 2011).

An expected average accretion of ~ 100 mg/day has been assumed by the Institute of Medicine from the available data. Largely, these studies imply that pregnant women do not provide much calcium or other minerals to their foetuses until the third trimester, when the peak rate of calcium transfer exceeds 300 mg/day on average (Kovacs, 2016). Data for lactating women and their babies are more variable, but suggest that the

average calcium requirement is more acceptable, at ~ 200 mg/day during the first 6 months, and ~ 120 mg/day during the second 6 months. All of these values, from 300 mg/day during the third trimester to 120 mg/day during late lactation, may seem achievable given normal intake of calcium and normal efficiency of intestinal calcium absorption. However, fractional calcium absorption is normally ~ 25% of intake in healthy adults who consume adequate calcium (Hunt and Johnson, 2007).

If average competence of intestinal calcium absorption were depended upon, the pregnant woman would need an extra 1,200 mg/day intake in the third trimester, and lactating women would also need an extra 800 mg daily intake during the first 6 months and 480 mg daily during the second 6 months. These average calcium requirements during pregnancy and lactation are data from the United States National Health and Nutrition Examination Survey (NHANES) and Statistics Canada, in which actual calcium intakes have been established for American and Canadian women. There is no such equivalent data available in Nigeria.

Between ages 18 and 50, the 50th percentile for calcium intake ranges from 800-1,000 mg/day in the two populations, for an anticipated fractional absorption of 200–250 mg/day. The 25th percentile of intake is 600–700 mg daily, while the 5th percentile is about 400 mg daily, in both populations (Institute of Medicine, 2011); and as such, if normal intestinal calcium absorption were to be depended upon, it would mean that most women do not have adequate calcium intake to meet the joint needs of their infants and themselves. Greater intakes are not compulsory in women who take sufficient calcium because of several changes that occur during pregnancy and lactation. During pregnancy, the proficiency of intestinal calcium absorption increases

to meet the foetal requirement for calcium, although during lactation, skeletal resorption intensifies to make calcium available to milk.

The reason for these different adaptations being invoked during these two reproductive periods is unknown. These adaptations that are mediated by hormones normally meet the daily mineral requirements of the foetus and infant without unpleasant long-term consequences to the maternal skeleton. Though, exceptions do occur, particularly in women with consistently very low intakes of mineral, or preexisting disorders which cause skeletal fragility (Kovacs, 2014).

2.3 Skeletal and Mineral Physiology during Pregnancy

As noted in the introduction, pregnancy requires that a woman deliver by term to her baby an average of 30 g calcium, 20 g phosphorus, and 0.8 g magnesium. Approximately 80% or more of these amounts are provided during the third trimester. Hence, any adaptations to meet the foetal requirement for mineral are maximally needed during the third trimester. It may be expected that there would be increased intestinal calcium absorption, decreased renal calcium excretion, and increased skeletal resorption of calcium.

Though, the main adaptation is a twofold increase in intestinal calcium absorption. The kidneys dissipate calcium in the urine rather than preserving it, and there is usually at most only a very small contribution of calcium from skeletal resorption. Pregnant rats and mice have been commonly used to model maternal adaptations in mineral homeostasis during pregnancy, while pregnant ewes and pigs have been studied to a lesser extent (Kovacs, 2016). The rat has a very short gestation period (22 days) and

may produce 6–12 foetuses in each litter. The calcium requirements on the pregnant rat are proportionately more than in women who on average bear a singleton during a far more extended gestational period. The pregnant rat supplies 12 mg calcium per foetus between day 17 of gestation and term, or greater than 95% of mineral during the corresponding rodent's third trimester.

Extrapolating from these studies, the pregnant mouse likely delivers more than 95% of the mineral content to its foetuses during the last 5 days of a 19-day gestation. The proportional demand on pregnant rodents is even more striking when it is considered that the amount of calcium transferred to a litter of rat foetuses each hour represents ~ 80% of the amount of calcium present in the maternal circulation, whereas a human foetus takes only ~ 5–10% of the quantity of calcium present each hour in the mother's circulation. As a result, pregnancy is extremely more capable of causing hypocalcaemia and secondary hyperparathyroidism in rodents than in women (Kovacs, 2014).

2.3.1 Changes in mineral ions, calciotropic and phosphotropic hormones

Changes in serum calcium, phosphorus, and calciotropic hormone levels during human pregnancy are shown in figure 1. Important differences among human, rat, and mouse pregnancies are listed in table 1.1.

Table 2.1: Key differences in Mineral Physiology of Human and Rodent Pregnancy

	Human	**Rat**	**Mouse**
Serum calcium	Low	Low	Normal
Ionised calcium	Normal	Low	Normal
Phosphorus	Normal	Normal	Normal
PTH	Low or low-normal*	Increased	Low or normal
Calcitonin	Increased	Increased	Increased
Calcitriol	Increased	Increased	Increased
Intestinal calcium absorption	Increased	Increased	Increased
Urinary calcium excretion	Increased	Increased	Increased
Change in bone mass	Slight loss	Small loss	Increased or no change

*Women with low calcium or high phytate intakes have normal or increased PTH. (Kovacs, 2016)

In some animal studies, it has been reported that serum calcium decreases during late pregnancy in rats, guinea pigs, sheep, goats, cows, and white-tailed deer. It has commonly been measured without correcting for any change in the protein or albumin concentration, even though a progressive 10–25% fall in protein and albumin has been found in pregnant rats, guinea pigs, and sheep (Dubreuil *et al*., 2005; Kovacs, 2016). The decline in serum albumin triggers a decrease in serum calcium; however, a decrease in serum calcium does not essentially point towards a change in ionized calcium, which is the physiologically significant fraction of extracellular calcium.

A report about rats consuming a 0.75% calcium diet discovered that the ionized calcium gradually increased while the serum calcium was decreasing (Quan-Sheng and Miller, 1989). Whereas rats given a 0.4% calcium diet were found to have a significant fall in the ionized calcium during late pregnancy (Boass *et al*., 1997). The observed drop in serum calcium during pregnancy usually correspond to the effect of decrease in the albumin-bound fraction, while the ionized calcium may be unchanged unless a reduced calcium diet is consumed (such as 0.4% calcium). A conflicting setback in available rat studies during pregnancy and lactation is the calcium content of diet. The 1976 and consequently reformulated 1993 standard rodent diets of the American Institute of Nutrition indicate a 0.50% content of calcium.

In past studies, it is usual to see diets with 0.4, 0.45, and 0.5% calcium. Though, a 1% calcium diet is now more frequently used in rats, and in most studies of mice, which influences such parameters as serum calcium, parathyroid hormone (PTH), bone turnover (Kovacs, 2016). Mice may differ from rats in their blood calcium parameters in pregnancy. In several studies in which wild-type mice consumed a standard1%

calcium diet, there was no difference in the ionized calcium or the serum calcium during pregnancy (Fudge and Kovacs, 2010; Kirby *et al.,* 2011; Kirby *et al.,* 2013). Physiologically significant hypocalcaemia can happen during pregnancy when the foetal requirement for calcium surpasses the mother's ability to support her own serum calcium. This might be particularly accurate in rats where the hourly foetal demand for calcium almost equals the amount of calcium present in the maternal circulation.

Consistent with this, larger litter number and weight are correlated with a lower serum calcium in the mother, whereas pregnant rats fed a low-calcium (0.1%) diet suffered a marked decrease in both serum calcium and ionized calcium in the last number of days before delivery. A low-calcium diet in the same way triggered hypocalcaemia in pregnant ewes compared with consumption of a usual and a high-calcium diet (Kovacs, 2016).

Unexpected deaths towards late pregnancy from apparent serious hypocalcaemia have occurred in rats made severely vitamin D deficient, and in Vdr null (Kovacs *et al.,* 2005; Fudge and Kovacs, 2010) and Cyp27b1 null mice (Gilles *et al.,* 2015) sustained on a normal 1% calcium diet. Serum phosphorus stays normal during pregnancy in rats and mice (Kirby *et al.,* 2011; Kirby *et al.,* 2013).

In humans, the first studies in pregnant women found a progressive 5–10% fall in serum calcium in which the mean value dropped below the normal range, thus suggesting biochemical hypocalcaemia (Kovacs, 2016). These findings corroborated with what had previously been reported in animal models, and proposed that the human foetus removes calcium from the maternal circulation in sufficient amounts to provoke

maternal hypocalcaemia and secondary hyperparathyroidism. Studies later discovered that serum albumin declines during pregnancy as a result of normal expansion of the intravascular volume, in so doing causing a fall in the albumin-bound fraction of calcium. The fall in the albumin-bound fraction of serum calcium is physiologically insignificant. As it became feasible to determine the ultrafiltrable fraction of serum calcium (complexed and ionized calcium, excluding albumin-bound calcium); there was no difference when prepregnancy and pregnancy measurements were matched. Recent advances led to the possibility of determining the ionized calcium with ion-specific electrodes.

Both cross-sectional and longitudinal studies have both revealed that the ionized calcium does not vary during human pregnancy, although the mean serum calcium concurrently declines ~ 10% into the hypocalcaemic limit. Albumin-corrected serum calcium is a logical alternative for the ionized calcium, since a number of longitudinal and cross-sectional studies have shown that it stays stable through all three trimesters, even though there is a concomitant fall in serum albumin (Nakayama *et al.,* 2011; Møller *et al.,* 2013; Roth *et al.,* 2013).

Women carrying twin pregnancies do not have impaired ability to maintain a normal serum calcium, as demonstrated in a comparative study of women carrying twin as opposed to singleton pregnancies (Nakayama *et al.,* 2011). Serum phosphorus does not vary in most findings during the course of human pregnancy, even in twin pregnancies (Nakayama *et al.,* 2011; Møller *et al*., 2013). Renal tubular reabsorption of phosphorus and serum magnesium, similarly shows no variation during pregnancy. To summarise, it would suffice it to say that in the course of normal human pregnancy, the expansion

of the intravascular volume brings about a fall in serum albumin and, in so doing, the serum calcium. Though, the ionized calcium and albumin-corrected serum calcium are not altered, substantiating that the physiologically important circulating fraction of calcium concentration stays unchanged. Either the ionized calcium or the albumin-corrected serum calcium may be reliably used to assess adequacy of the blood calcium concentration during pregnancy. Animal models show a similar albumin-related fall in serum calcium, whereas both the serum and ionized calcium levels appear to stay constant in pregnant mice, likely because albumin does not decline. Ionized calcium is more likely to fall in rodents when challenged by larger litters, low calcium diets, or disorders of vitamin D physiology (Kovacs, 2016).

2.3.2 The role of calcium in pregnancy

It has been well established that calcium plays an essential role in both the development and maintenance of bone health. Although osteoporosis is a condition of childhood that presents in older age, the importance of calcium homeostasis and optimum calcium intake throughout the lifespan is crucial in the prevention of senile osteoporosis (Hacker-Thompson *et al.,* 2012). There is evidence suggesting that maternal calcium intake can affect the bone development of the foetus and its future skeletal growth. Thus, it is essential that women of child-bearing age are educated on the importance of meeting their calcium requirements.

Women of child-bearing age will meet their own needs and those of their infants, if they regularly consume adequate amounts of calcium (1,000 mg/day). Additional calcium supplementation during pregnancy appears to have the greatest impact in

women who chronically consume <500 mg calcium/day, demonstrating the importance of adequate calcium intake before pregnancy begins (Hacker-Thompson *et al.,* 2012).

Calcium and phosphate play many essential roles in the body. To accomplish these functions, serum levels need to be maintained in a certain range with a complex interplay of hormones involved to maintain a balance (Fukumoto, 2014). The most important factors involved in the balance are vitamin D and parathyroid hormone. Calcitonin is a hormone secreted from thyroid and is involved in calcium homeostasis, but far less important than parathyroid hormone. It primarily has calcium-lowering effect (Kumar, 2009). Calcium homeostasis is a function of dietary intake, intestinal absorption, skeletal accretion and resorption, and urinary excretion.

The key organs involved in the changes in calcium metabolism during pregnancy include the maternal parathyroid gland, the maternal intestine, and the foetoplacental unit (Kovacs, 2008). Pregnancy and lactation cause a substantial increase in demand for calcium that is met by different maternal adaptations within these periods. This adaptation essentially involves increased intestinal calcium absorption, which more than doubles during pregnancy and maternal skeleton resorption to provide most of the calcium content of breast milk during lactation (Kovacs, 2008). The pregnant woman's body provides 25 to 30 g of calcium to support the developing foetal skeleton. Much of the foetal calcium demand occurs in the third trimester of pregnancy, when the foetal skeleton undergoes mineralisation (Holick *et al*., 2012).

Within the placenta, calcium is a very important secondary messenger that takes part in signal transduction pathways vital to proper placentation and foetal development. By

the the end of the third trimester, 25 to 30 g calcium would have been accreted from the mother to the foetus across the foeto-maternal unit. Foetal calcium regulation takes into consideration maternal calcium homeostasis as well as placental transport capacity which hinge on the existence and the function of many different placental calcium channels and transporters (Papadopoulou *et al.,* 2014). Transient receptor potential cation channel subfamily (VTRPV6) (a membrane calcium channel) and calbidin are some of the factors involved in placental calcium transport that have also been investigated in pregnancy pathologies such as preeclampsia, Intrauterine growth restriction and gestational diabetes mellitus (Strid *et al*., 2003).

After delivery, neonates go through a dramatic but transient decline in serum calcium levels, typically by 24 to 48 h of life, pending the maturation of the postnatal mechanisms of calcium homeostasis (Kovacs, 2011). The calcium sensing receptor (CaSR) belongs to the superfamily of G protein-coupled cell surface receptors (GPCRs) and its role in humans is the maintenance of the normal serum calcium concentration primarily through the regulation of parathyroid hormone (PTH) synthesis and secretion from the parathyroid glands.

According to the cells expressed and the existing cascades, CaSR regulates cytosolic Caþþ signaling, hormone secretion or inhibition and takes part in many other processes, like cell proliferation and differentiation (Chakravarti *et al.,* 2012). Specific inactivating or activating mutations of the CaSR gene are related with hereditary hypercalcaemic or hypocalcaemic disorders, respectively; known as Autosomal Dominant Hypocalcaemia (ADH), Neonatal Severe Hyperparathyroidism (NSHPT) and Familial Hypocalciuric Hypercalcaemia (FHH). In addition, apart from

pathological conditions arising from CaSR mutations, common genetic variations in the CaSR gene appear to affect serum calcium levels, as reported in a previous study (O'Seaghdha *et al.,* 2010). However many regulatory factors are implicated in calcium transfer through the foetomaternal unit and several pathophysiologic mechanisms are involved in the disturbances of calcium homeostasis in pregnancy pathologies.

2.3.3 Low or high calcium intake

Pregnancy is normally a state of absorptive hypercalciuria with suppressed PTH, which implies that for most women calcium is absorbed in excess of maternal and foetal requirements. But then again, PTH does not fall and may rise above normal in women with low dietary intakes of calcium or high intake of phytate (which blocks calcium absorption). However, in some women with chronically low calcium intakes, the efficiency of intestinal calcium absorption may be greater than normal, as suggested by a study in which most women with a dietary intake of less than 420 mg were in a positive calcium balance in all three trimesters. A lower calcium intake is more likely to induce bone loss during pregnancy and has been associated with increased risk of osteoporosis during pregnancy (Kovacs, 2015).

Whether the mother obtains the needed mineral from her diet or skeleton may be of no consequence to the foetus, although a randomized trial found that 2g of supplemental calcium versus placebo improved the bone mineral content (BMC) of babies whose mothers were in the lowest quintile of dietary calcium intake (< 600 mg/day). Significant maternal hypocalcaemia impairs the delivery of calcium to the foetuses, who may develop secondary hyperparathyroidism, skeletal demineralization, and fractures, as has occurred in cases of poorly treated maternal hypoparathyroidism

during pregnancy (Kovacs, 2014). The lowest quintile of maternal calcium intake is also associated with increased risk of preeclampsia, whereas calcium supplementation reduces that risk. In contrast to low calcium intake, excessively high intake will create similar effects as primary hyperparathyroidism in pregnancy: increased net absorption of calcium, maternal hypercalcaemia, increased maternal to foetal transfer of calcium, and suppression of the foetal parathyroids (Kolnick *et al.*, 2011; Borkenhagen *et al.*, 2013). Neonatal hypoparathyroidism has resulted from women consuming 3–6 g elemental calcium daily to treat nausea of pregnancy (Kolnic *et al.*, 2011; Borkenhagen *et al.*, 2013).

2.4 Hormones Secreted in Pregnancy

During the course of pregnancy, many of the hormones secreted in the non-pregnant state are usually increased in production as a result of increased metabolism of the mother, they include: parathyroid hormone, thyroxine, relaxin, calcitonin and 1, 25 Dihydroxycholecalciferol (Vitamin D), progesterone, oestrogen, human chorionic gonadotropin, thyrotropin, prolactin, glucocorticoids, aldosterone and fibroblast growth factor 23 (Fukumoto and Shimizu, 2011) amongst others.

Although there is a marked decrease in the secretion of follicle stimulating hormone and lutenising hormone from the pituitary gland as a result of the inhibitory effect of oestrogens and progesterone being secreted by the placenta (Barrett *et al.*, 2010); for the purpose of this work, we discussed the parathyroid hormone, calcitonin and 1, 25 dihydroxycholecalciferol and briefly the fibroblast growth factor 23 because it is novel in its discovery as a phosphotropic hormone.

Sometimes endocrine disruptors may disturb the balance of these hormones and calcium metabolism (Kim *et al.,* 2013). In Nigeria, there is extensive use of pesticides with little or no regulation of their use; cosmetics, diet and household items containing endocrine disrupting chemicals with deleterious effects on reproductive and endocrine homeostasis, and consequently on reproduction/fertility (Avidime *et al.,* 2011a; Avidime *et al.,* 2011b). This could lead to reduced levels of the hormones.

2.4.1 Calciotropic and phosphotropic hormones

Fibroblast growth factor 23 (FGF23) is produced by bone and reduces serum phosphate by inhibiting proximal tubular phosphate reabsorption and intestinal phosphate absorption. Excess actions of FGF23 cause several kinds of hypophosphataemic rickets/osteomalacia, while deficient actions of FGF23 result in hyperphosphataemic tumoral calcinosis. In addition, FGF23 has been shown to prevent the development of hyperphosphataemia during the progression of chronic kidney disease-mineral and bone disorder.

Epidemiological studies have indicated that high FGF23 levels are associated with unfavorable events including higher mortality, cardiovascular events, progression of chronic kidney disease (CKD) and fracture; however, these associations are not observed unequivocally and it is not evident why they are present. While, FGF23 has been shown to be a hormone that regulates phosphate metabolism, it remains to be established whether FGF23 has roles other than regulating mineral homeostasis (Fukumoto and Shimizu, 2011).

2.4.2 Vitamin D hormone

Vitamin D is a prohormone that is derived from cholesterol. The nutritional forms of vitamin D include D_3 (cholecalciferol), which is generated in the skin of humans and animals, and vitamin D_2 (ergocalciferol), derived from plants. Both forms are absorbed in the gut and used by humans (Mulligan *et al.*, 2010).

2.4.2.1 Sources of vitamin D

Vitamin D occurs naturally in fish and some plants, but is not found in significant amounts in meat, poultry, dairy products (without fortification), or the most commonly eaten fruits and vegetables. In Nigeria, common sources of Vitamin D include wild salmon (Titus) (99.223 g) provides 600-1000 IU; farmed salmon has approximately 25% of this amount per serving (Lu *et al.*,2007) while same amount of mackerel, sardines, or tuna (*kote*) fish provides 200-300 IU, cod liver oil (1 tsp) provides 600-1000 IU. In the United States, the major dietary sources of vitamin D are fortified foods (Mulligan *et al.*, 2010). For example, 226.8g of fortified milk, orange juice, or yoghurt, 85.1 g of fortified cheese, or a serving of fortified breakfast cereal each provides 100 IU of vitamin D (Ko *et al*., 2008).

2.4.2.2 Discovery of vitamin D

A lot of studies have been done on vitamin D, the Nobel prize for chemistry in 1928 was given to Adolf Windaus for his studies on the constitution of the sterols and their connection with vitamins. His corroboration with Rosenheim and Hess along with some other researchers led to the discovery of this vitamin (Wolf, 2004). Further studies of vitamin D in the diet revealed the discovery of 25-hydroxyvitamin D [25(OH)D] and the eventual discovery of the hormonal form of vitamin D; 1,25-

dihydroxyvitamin D [1,25$(OH)_2$D)] by a number of investigators (Hollis and Wagner, 2013).

2.4.2.3 Physiology of vitamin D

Because vitamin D is a prohormone, it is converted to its active form 1, 25 (OH) D_2. The ultraviolet rays of the sun impacts on the skin and aids the conversion of 7-dehydrocholesterol found in the subcutaneous fat to pro-vitamin D, which goes through thermal isomerization to vitamin D_3 and vitamin D_2 (Ross *et al.*, 2011). Vitamin D_3 and D_2 are not naturally present in the food consumed by the majority of the African populace. Moreover, the heavily pigmented skin of the sub-saharan African by the sun to shield us from its cancerous affront, at the same time denies us of our skin's ability to manufacture vitamin D with the aid of the same solar energy.

Vitamin D, also called calciferol, consists of a set of fat-soluble seco-sterols. The two main forms are vitamin D_2 and vitamin D_3. Vitamin D_2 (ergocalciferol) is essentially human-made and used to fortify food while vitamin D_3 (cholecalciferol) is manufactured in the dermis of humans from 7-dehydrocholesterol and is ingested in the food through the consumption of animal-based diets. Vitamin D_3 and vitamin D_2 are manufactured commercially and obtained in food supplements and fortified foods. D_2 and D_3 are similar in form except in their side chain structure. This dissimilarity does not alter metabolism (i.e., activation), and the two varieties act as prohormones. Table 2.2 shows the different nomenclatures by which vitamin D is known.

Table 2.2: Nomenclature of vitamin D precursors and metabolites

Common Name	Clinical Name	Abbreviation	Comments
7-Dehydrocholesterol	Pro-vitamin D_3	7DHC	Lipid in cell membranes
Cholecalciferol	Pro-vitamin D_3		Photosynthesized in skin or diet
Ergocalciferol	Pre-vitamin D_2		Obtained from diet. Equivalent to vitamin D_3 as precursor for active vitamin D
Calcidiol	25-Hydroxyvitamin D	25[OH]D	Best reflects vitamin D status
Calcitriol	1,25-Dihydroxvitamin D	1,25[OH]D_2	Active form of vitamin D, tightly regulated

Adapted from Alshahrani and Aljohani, 2013

Studies have shown that once they have been activated, the D_2 and D_3 demonstrate the same responses in the body, and the effectiveness associated to the ability to treat vitamin D–deficiency rickets is the same (Jurutka et al., 2001). Some animal studies have suggested that vitamin D_2 is not as toxic as vitamin D_3, although this has not been proven in humans. Figure 2.1 illustrates the steps involved in the activation of vitamin D from the diet and dermal synthesis.

Vitamin D_2/D_3 is regarded as biologically inactive until it undergoes two enzymatic hydroxylation reactions as seen in Figure 2.3 and Figure 2.4. The first one is initiated in the liver, facilitated by the 25-hydroxylase which forms 25-hydroxyvitamin D (25OHD). The second reaction occurs in the kidney, facilitated by 1α-hydroxylase (CYP27B1), which transforms 25OHD to the biologically functional hormone, calcitriol (1,25-dihydroxyvitamin D). The 1α-hydroxylase gene is similarly expressed in a number of extra-renal tissues, although its involvement in calcitriol formation in these tissues has not yet been estabilished (Ross *et al.,* 2011).

The most important circulating form of vitamin D is 25OHD which is the precursor of calcitriol, it is bound to a specific plasma carrier protein, vitamin D binding protein (DBP) in circulation. This DBP is also responsible for transporting vitamin D and calcitriol. Vitamin D regulates absorption of calcium from the gastrointestinal tract as well as its excretion from kidney. Several hormones such as parathyroid hormone mainly function through conversion of vitamin D to its active metabolite (1,25-hydroxycholecalcipherol) in the kidneys, which regulates intestinal absorption, renal excretion, and exchange between the extracellular fluid and bone (Levine *et al*., 2014).

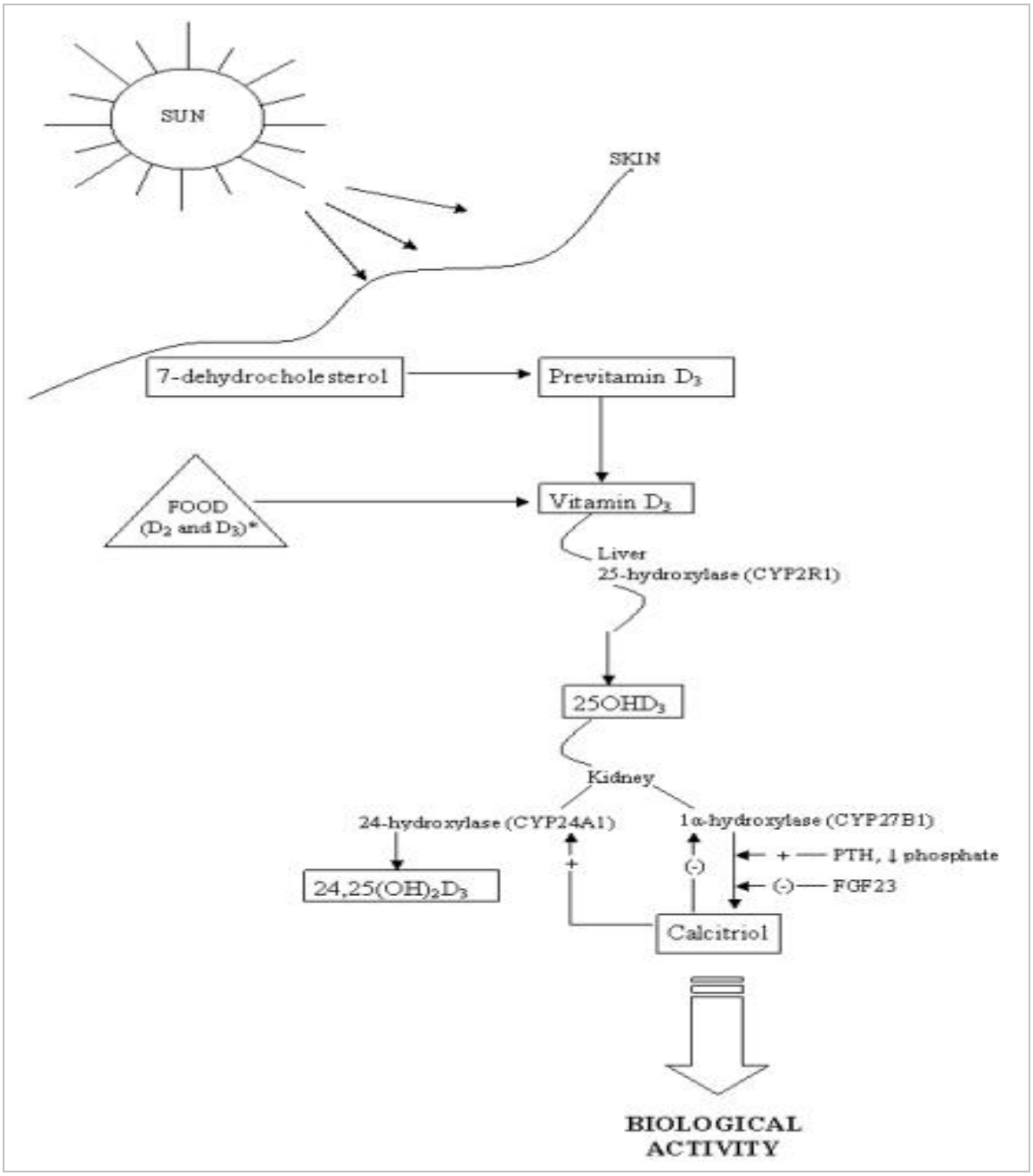


Figure 2.2: Overview of vitamin D synthesis, intake, and activation (Ross *et al.*, 2011)

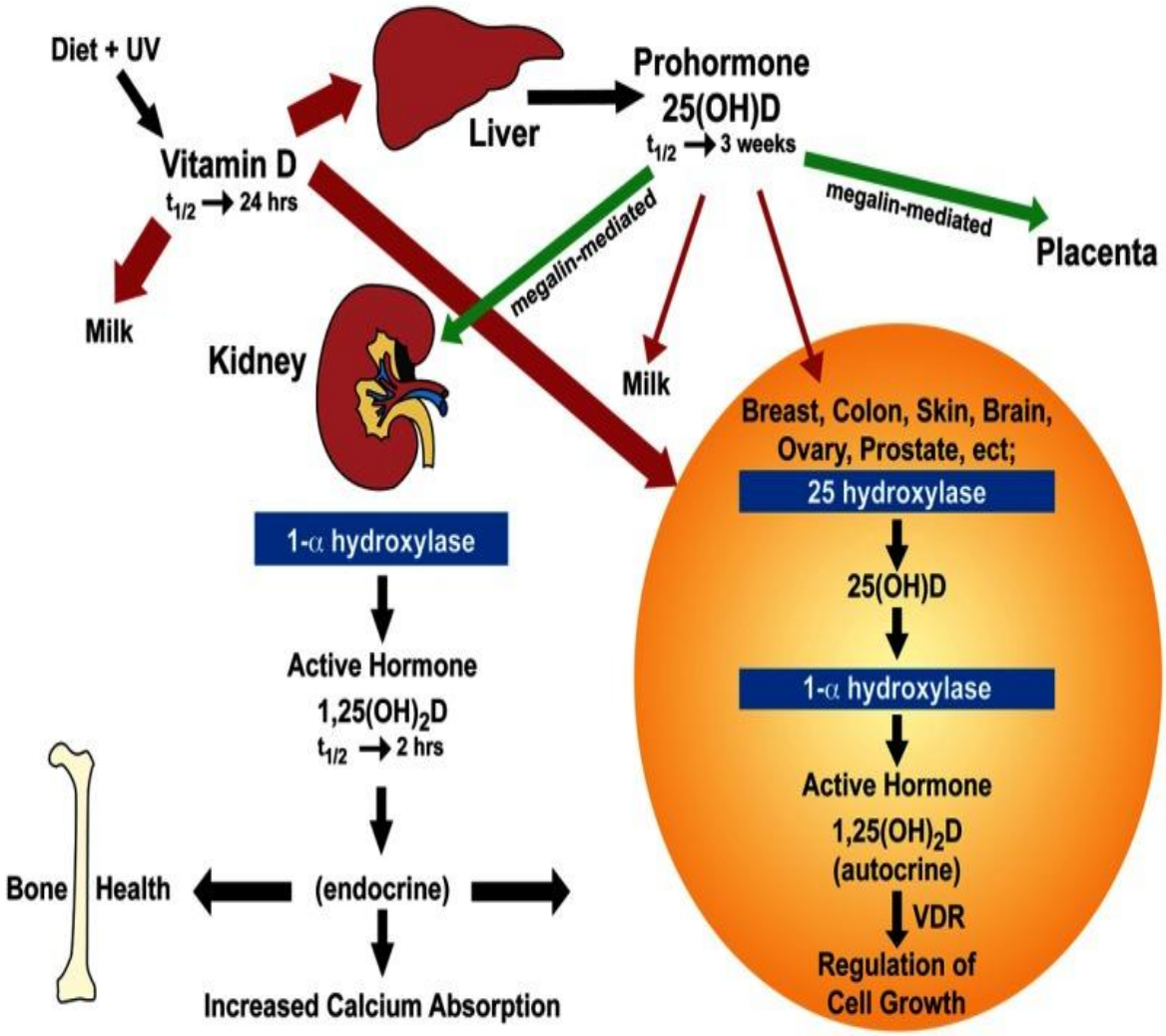


Figure 2.2: Vitamin D and tissue homeostasis

Diagram of the metabolic processes providing vitamin D and its metabolites to various tissues of the body. Tissue distribution of vitamin D and 25(OH)D based on simple diffusion (red arrows) or endocytosis (green arrows). Endocytosis requires the tissue-specific megalin-cubilin system, whereas simple diffusion is primarily controlled by the dissociation constant of the vitamin D compound for the VDBP. Bolder red lines indicate greater diffusion rates due to a higher dissociation constant. $t_{1/2}$, half life. (Hollis and Wagner, 2013)

2.4.2.4 Reference ranges for vitamin D

A unique aspect of vitamin D as a nutrient is that it can be synthesized by the human body through the action of sunlight. These dual sources of vitamin D make it exigent to develop dietary reference intake values, though the range of serum 25-hydroxyvitamin D is from 20 to 50 ng/ml (Ross *et al.*, 2011). Table 2.3 shows the dietary reference intakes that makes for adequate vitamin D intake

Table 2.3: Vitamin D dietary reference intakes (DRIs) for adequacy (amount/day)

Life Stage Group	Adequate Intake	EAR	RDA
Infants			
0 – 6 months	400IU (10 μg)	-	-
6 – 12 months	400IU (10 μg)	-	-
Children			
1 – 3 years	-	400IU (10 μg)	600 IU (15 μg)
4 – 8 years	-	400IU (10 μg)	600 IU (15 μg)
Males			
9 – 13 years	-	400IU (10 μg)	600 IU (20 μg)
14 – 18 years	-	400IU (10 μg)	600 IU (20 μg)
19 – 30 years	-	400IU (10 μg)	600 IU (20 μg)
31 – 50 years	-	400IU (10 μg)	600 IU (20 μg)
51 – 70 years	-	400IU (10 μg)	600 IU (20 μg)
> 70 years	-	400IU (10 μg)	800 IU (20 μg)
Females			
9 – 13 years	-	400IU (10 μg)	600 IU (20 μg)
14 – 18 years	-	400IU (10 μg)	600 IU (20 μg)
19 – 30 years	-	400IU (10 μg)	600 IU (20 μg)
31 – 50 years	-	400IU (10 μg)	600 IU (20 μg)
51 – 70 years	-	400IU (10 μg)	600 IU (20 μg)
> 70 years	-	400IU (10 μg)	800 IU (20 μg)
Pregnancy			
14 – 18 years	-	400IU (10 μg)	600 IU (20 μg)
19 – 30 years	-	400IU (10 μg)	600 IU (20 μg)
31 – 50 years	-	400IU (10 μg)	600 IU (20 μg)
Lactation			
14 – 18 years	-	400IU (10 μg)	600 IU (20 μg)
19 – 30 years	-	400IU (10 μg)	600 IU (20 μg)
31 – 50 years	-	400IU (10 μg)	600 IU (20 μg)

EAR = Estimated Average Requirement, RDA = Recommended Dietary Allowance, IU = International Unit (Adapted from Dietary Reference Intakes for Calcium and Vitamin D, 2011)

2.4.2.5 Vitamin D and the physiology of pregnancy

Animal studies have shown an increased serum calcitriol concentration sometimes up to seven times during the course of normal pregnancy (Kirby *et al.,* 2011; Kirby *et al.,* 2013). In rats and mice, vitamin D binding protein levels were observed to be raised to some extent, while there was a slight decrease in rats and guinea pigs (Kirby *et al.,* 2013). It can then be extrapolated that in these species, concentration of calcitriol is significantly raised towards the third trimester. In rats, this increase in total calcitriol has also been shown to only happen during the last days of pregnancy (Quang-Sheng and Miller, 1989; Boass *et al.,* 1997), while in guinea pigs free calcitriol was elevated prior to the third trimester and also before the total calcitriol rose in concentration (Verhaeghe *et al.,* 1994).

This also corresponds to the time foetal mineral accretion occurs rapidly. Increased maternal calcitriol levels have been correlated with larger litters (Boass *et al.,* 1997). In a Nigerian study using mice, there was a significant decrease in the average weight, length and number of litters produced which was attributed to giving the mice oral cholecalciferol at high doses (Ogamba *et al.,* 2011). Reports have shown renal Cyp27b1 (the enzyme, 1 – alphahydroxylase which catalyses the hydroxylation of calcifediol to calcitriol) to be upregulated up to five times when in vitro analysis of homogenates obtained from guinea pig kidneys were done (Kubota *et al.,*1982). Therefore, the maternal kidneys are probably the source of increased production of calcitriol just as it is in the non-pregnant adult (Kovacs and Ralston, 2015).

Other potential sources of calcitriol due to the presence of Cyp27b1 in their tissues are the maternal decidua, placenta and the foetus (Gray *et al.,* 1981; Glorieux *et al.,* 1995).

In a review by Yates *et al.*, 2017, they discussed the possibility of glucocorticoids helping in the regulation of vitamin D in the placenta since it is involved in cellular proliferation, differentiation, vascular function and immune regulation. It is important for the decidualisation of the myometrium in the female, modulation of maternal immune function and foetal bone formation The placenta is a vital source of vitamin D which regulates its metabolism through the vitamin D receptor (VDR), although, the pathway through which VDR regulates trophoblast function is not fully understood (Nguyen *et al*., 2015).

It is likely that a blend of factors such as rising concentrations of PTHrP, estradiol, calcitonin, and placental lactogen, could add up to upregulate Cyp27b1 during pregnancy; instead of one factor being specifically being in control. It may also be that there is an unknown factor which stimulates the increase of Cyp27b1 during pregnancy (Kovacs, 2016).

Human studies have shown outcomes similar to those involving animal models. Some of such studies have discovered a threefold increase in serum calcitriol levels from the commencement of the first trimester and attains the peak level by the third trimester (Møller *et al.,* 2013); while other studies have established a gradual increase in free and bound calcitriol which is maintained throughout pregnancy (Bikle *et al*., 1984). Free calcitriol has also been reported to be increased in the third trimester in another investigation, though the more moderate increase of up to 40% in vitamin D binding protein during pregnancy, and the decrease in serum albumin, signifies that free calcitriol is possibly increased in the three trimesters of pregnancy (Hollis *et al.,* 2011; Zhang *et al.,* 2014).

In a large cross-sectional study, serum calcitriol was increased three times in women who had singleton and twin pregnancies, with no dissimilarity between the two groups (Nakayama *et al.*, 2011). No difference was also observed in the vitamin D binding protein and albumin levels, meaning that singleton and twin pregnancies probably resulted in the same increases in free calcitriol (Nakayama *et al.*, 2011), while in another smaller longitudinal study, they found no difference in serum calcitriol involving twin and singleton pregnancies all through the pregnancy.

A substantial curiosity about the marked pregnancy-related increase in calcitriol is that it occurs while PTH is often suppressed to low levels, which suggests that PTH is not responsible for the upregulation of Cyp27b1. An additional analysis in pregnant women found that the increased calcitriol did not correlate with PTH, prolactin, estrone, estradiol, estriol, or human placental lactogen (Reddy *et al.*, 1983). Conflicting data exists as to whether there is change in serum 25OHD concentration during human pregnancy. In a number of sizeable investigations there was no significant change (Cooper *et al.*, 2016; Roth *et al.*, 2013; Wagner *et al.*, 2013) or even a slight increase (Morley *et al.*, 2006). Some longitudinal experiments have found a small but significant fall in total 25OHD or calculated free 25OHD values (Zhang *et al.*, 2014) in the course of pregnancy.

Pattern of eating habits, seasonal and late pregnancy adaptations in sunlight exposure and smaller sample sizes may have influenced these studies (Kovacs, 2016). Most of the data point to the fact that maternal 25OHD is not significantly depleted into the foetal circulation, or exhausted by conversion into calcitriol. It was discovered that the maternal 25OHD was also steady throughout all the trimesters in singleton and twin

pregnancies in a large cross-sectional investigation; but, the 25OHD level was 30% reduced in women with twin pregnancies during the three trimesters (Nakayama *et al.,* 2011). It is not known whether, this difference was due to the twin babies increased demand on the maternal supply of 25OHD, or due to less sunlight exposure or vitamin D consumption in women carrying twins.

The absence of a gradual change in 25OHD during pregnancy, specifically a progressive change between mean values in women carrying singleton pregnancy as against twin pregnancy, makes it unlikely that twin pregnancy signifies depletion on the maternal supply of 25OHD. In a smaller longitudinal study, no disparity in 25OHD levels between women carrying twins versus singletons was reported, it may be suggested that the larger cross-sectional study was confounded by innate differences between both groups of pregnant women rather than expounding an effect of twin pregnancy. There are no data from women bearing triplets (Kovacs, 2016).

Other physiological functions of vitamin D have been suggested as discussed in the review by Schogl and Holick, (2014), some of which includes strong evidence suggesting that $1,25(OH)_2D$ plays a role in neuroprotection by regulating the production of nerve growth, decreasing L-type calcium channel expression, regulating the toxicity of reactive oxygen species, and neurotrophic factors such as nerve growth factor, glial cell-derived neurotrophic factor; and nitric oxide synthase (Chabas *et al.,* 2008; Marini *et al.,* 2010). Also, vitamin D and its metabolites are concerned with other neuroprotective mechanisms comprising amyloid phagocytosis and clearance, (Mizwicki *et al.,* 2013), and vasoprotection. Vitamin D plays a protective function in the cardiovascular system by downregulating the renin–angiotensin–aldosterone

system, (Forman *et al.,* 2010), cardiac remodeling (Chen *et al.,* 2011), regulating the endothelial response to injury and blood coagulation by increasing thrombus formation and tissue factor activity (Caprio *et al.,* 2012; Sypniewska *et al.,* 2013).

2.4.2.6 Vitamin D deficiency, disorders and disease

Wagner *et al.,* in their 2015 review reported that there are few studies that have explored the association linking vitamin D status to preterm birth. Also, those with lower 25(OH)D concentrations had higher odds of preterm birth. These findings suggest that, while timely intervention with vitamin D supplementation throughout pregnancy can influence future risk of preterm births, especially in Hispanic women, those diagnosed with vitamin D deficiency during pregnancy may also profit from vitamin D therapy.

In an earlier study (Wagner *et al.,* 2013), they reported an association linking 25(OH)D concentration with preterm birth, and also took into account women that had spontaneous preterm birth and women who had hypertensive disorders of pregnancy and preeclampsia. In a cross-sectional study of 461 pregnant women in Nigeria, a prevalence of 29% vitamin D deficiency was found (Gbadegesin *et al.,* 2017), they also observed no differences between the groups with regards to pregnancy complications and preeclampsia. Another study with pregnant teenagers found serum vitamin D and 1,25-dihydroxyvitamin D concentrations to be within the normal range in the second and third trimesters (Sanchez *et al.,* 1997).

The custom of purdah and use of hijab among Muslim Nigerian women especially in the north may significantly disturb their vitamin D status, as a result of deprivation of

sunlight exposure (Lindsay *et al.,* 2012). Reports have shown that some muslim Nigerian women had a lower serum 25-hydroxyvitamin D [25(OH)D] concentration and it was also reported that pregnant hijab clad Nigerian women had lower plasma levels of calcium and phosphate and lower serum 25(OH)D levels than their nonpurdah Nigerian counterparts (Okonofua *et al.*, 1986; Glew *et al.*, 2010).

However, the 25(OH)D concentrations of the two groups of Nigerian women were still higher than that of Caucasian women in the UK. Though there is limited data on the calcium and vitamin D status of pregnant Nigerian women and subsequent pregnancy outcomes, it seems that they run the risk of deficiencies of vitamin D (Okonofua *et al*., 1986; Sanchez *et al*., 1997; Ojofeitimi *et al*., 2008; Glew *et al*., 2010). This could possibly have an effect on foetal bone development and might impact on the high prevalence of rickets among Nigerian children, also it was reported that some of these women risked adverse pregnancy outcomes as a result of inadequate micronutrient status in early pregnancy (Lindsay *et al.,* 2012; Lindsay *et al.,* 2014).

Studies have shown that calcitriol exerts immunomodulatory and anti-proliferative effects across autocrine and paracrine pathways and it has also been postulated that extensive actions of calcitriol also plays a possible role in defense or curative action in cancer and protracted disorders such as in auto-immune disorders (including type 1 diabetes), cardiovascular diseases, and infections (Holick et al., 2008). Asides the biological forms of vitamin D, there is a variety of analogues based on the vitamin D structure that have been manufactured for utilization as possible pharmacological agents. These agents are intended however for specific applications in research or

clinical therapy and are not food supplements or biosynthesized compounds (Ross *et al.*, 2011).

2.5 Parathyroid Hormone and Parathyroid Hormone-Related Protein

2.5.1 The discovery of parathyroid hormone

Many scientists have been involved in the discovery of the parathyroid hormone (PTH) such as Virchow who described the human parathyroids in 1963, with the first complete description being given by Sandstroem, a Swedish doctor in 1980 but its physiology was not understood until much later. Credit for the discovery of parathyroid hormone was given to the Canadian, James Bertram Collip in 1925, who used it along with his colleague Leitch to treat tetany successfully; while in the same year, it was named parathyrin (Kalra *et al*., 2013).

2.5.2 Physiology of parathyroid hormone

Parathyroid hormone/parat hormone or parathyrin (PTH), is first produced on the ribosomes as a preprohormone (preproparathyroid hormone), with a polypeptide chain of about 110 amino acids which is cleaved first to give a prohormone (proparathyroid hormone) containing 90 amino acids , then to the actual hormone in the endoplasmic reticulum and Golgi apparatus, and finally is packed in secretory granules of the cytoplasm of the cells of the parathyroid glands.

PTH has a final molecular weight of approximately 9500 Da, though there are smaller compounds that also display PTH activity with as little as 34 amino acids. Since the kidneys promptly eliminate the PTH hormone with 84 amino acids, these smaller compounds cause a great proportion of the hormonal action (Guyton and Hall, 2011).

PTH is the most significant hormone that regulates calcium balance; it gives a strong mechanism for regulating extracellular calcium and phosphate concentrations by controlling exchange of ions between the extracellular fluid and the bone, renal excretion and intestinal reabsorption (see figure 2.5). When the activity of PTH causes rapid absorption of calcium salts from the skeletal system, there is subsequent hypercalcaemia in the extracellular fluid; in contrast, hypofunction of the parathyroid glands could lead to hypocalcaemia, which is often associated with tetany (Guyton and Hall, 2011).

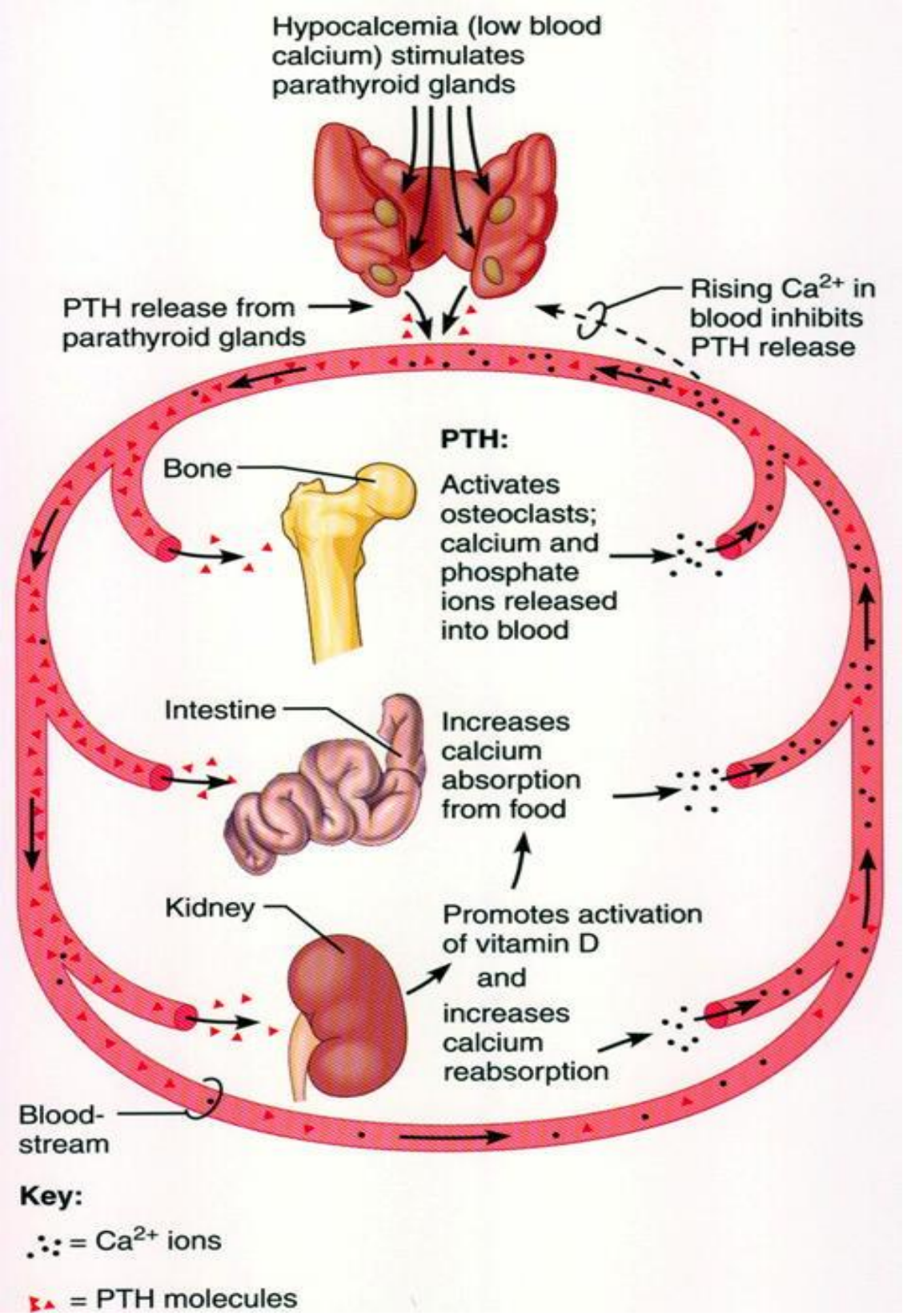


Figure 2.3: Circulation of parathyroid hormone (rci.rutgers.edu, 2017)

Asides its traditional function of regulation of blood calcium levels, there is mounting evidence to suggest that PTH does other activities that may be of significance to the advancement of extraskeletal disorders such as metabolic diseases comprising diabetes mellitus (Larsson *et al*., 2016). Strong correlation between circulating levels of glucose and PTH, and PTH level and insulin sensitivity have been estabilished in a number of studies (Norenstedt *et al.,* 2013).

The parathyroid hormone (PTH) group, together with PTH-related peptide (PTHrP), control calcium and bone homeostasis and a variety of hormonal processes through its receptors (the PTHRs). There are two PTHRs that have been recognized as PTH family ligands in mammals, assigned parathyroid hormone 1 receptor (PTH1R) and parathyroid hormone 2 receptor (PTH2R); while there are three in teleostei fish or other non-mammalian vertebrates, such as zebrafish, seabream and chicken (On *et al*., 2015). PTH1R arbitrates the actions of PTH and PTHrP in mammals. Human PTH2R, can be seen in the brain, pancreas, heart and kidneys, it is activated by PTH and tuberoinfundibular peptide of 39 residues (Liu *et al.*, 2016).

Preliminary records on PTH measurements in pregnant women used the earliest radioimmunoassays that generated unreliable and varying results, a lot of these investigations found significantly raised levels of PTH towards the end of pregnancy (Cruikshank *et al.,* 1980; Gillette *et al.,* 1982; Allgrove *et al*., 1985). Along with the concomitant decrease in serum calcium, this brought about the theory that pregnancy signifies a physiological state of secondary hyperparathyroidism. With further research, it was eventually realized that this assumption was flawed because the decrease in

serum calcium is triggered by a fall in albumin levels, while ionised calcium levels stay the same in pregnancy in humans (Kovacs, 2016).

In addition, these initial, insensitive PTH radioimmunoassays assessed several biologically inactive portions of PTH. The more sensitive and precise two-site immunoradiometric (IRMA) PTH assays are more accurate (Potts *et al*., 1995; Verhaeghe and Bouillon, 1992). In some cross-sectional analyses of European and North American women who were on a sufficient calcium diet, PTH is usually suppressed to the lesser end of the average range during all three trimesters of pregnancy (Seely *et al*., 1997; Davis *et al.*, 1998).

Other research from Asia, Europe and North America showed a mean PTH level that was supressed to the lesser end of the average range or lower in the course of the first trimester, and stayed suppressed or improved to mid-normal by late pregnancy (Møller *et al.,* 2013). There was no difference in serum PTH levels of women carrying twin pregnancies when compared with women carrying singleton pregnancies (Nakayama *et al.,* 2011). Due to the fact that PTH happens to be suppressed in the course of pregnancy, some recent textbooks of medicine maintain erroneously that pregnancy causes a condition of physiological hyperparathyroidism (Taylor and Badell, 2011).

In pregnant women from Africa and Asia, it has also been shown that there is PTH suppression using more recent assay techniques (Ainy *et al*., 2006; Shao *et al.,* 2012). Though some longitudinal and cohort studies discovered that PTH does not decrease with pregnancy; some other studies have shown that the PTH level does not decrease all through pregnancy; while in others it increased above the normal value (Madar *et*

al., 2009; Marwaha *et* al., 2011; Roth *et al.*, 2013; Schoenmakers *et al.*, 2013). The absence of suppression of PTH during pregnancy in women from Africa and Asia could be as a result of the local diets which are usually minimal in calcium or vitamin D, and have elevated phytate concentration that can impede calcium absorption. This reduced calcium ingestion or absorption could elicit compensatory secondary hyper-parathyroidism in the course of pregnancy.

It could be summarized that during pregnancy in humans, PTH levels in the blood normally decrease to lower concentrations initially, after which it goes back to the normal level by the end of the third trimester. This suppression might not take place (and secondary hyper-parathyroidism may result) in women who have diets particularly low on vitamin D, calcium, or high in phytate. As earlier mentioned, these diets are more peculiar to some parts of Africa and Asia (Kovacs, 2016).

2.5.3 Parathyroid hormone-related protein

In the year 1987, Parathyroid hormone-related protein (PTHrP) was cloned for the first time from human tumuors which produced humoral hypercalcaemia of malignancy. PTHrP is a prohormone which has been processed to quite a number of moieties, each of which has been hypothesised to have its own receptor and role. The NH_2-terminal region of PTHrP has some homology to PTH inside its first 13 amino acids, and an analogous configuration within the initial 34 amino acids, which allows it to activate the mutual PTH/PTHrP receptor and have almost duplicate functions as PTH (Kovacs, 2014).

The mid-molecular area of PTHrP has been revealed to encourage placental calcium transport in foetuses, but if it has any function in the mother, it remains unclarified. The COOH-terminal sections of PTHrP have been shown to impede osteoclast-mediated bone resorption *in vitro* and *in vivo*, and so it is believed that this section of PTHrP may help to avert undue resorption of the maternal skeleton in the course of pregnancy. PTHrP possibly arrives at the maternal circulation from a number of sources, comprising the parathyroids, placenta, mammary glands, and uterus (Kovacs, 2016). PTHrP has been presumed to circulate at augmented levels in animals that are pregnant since it occurs in humans, but amazingly, a number of measurements have been done in animals but there was no significant rise in PTHrP detected in one investigation of pregnant and non-pregnant mice (VanHouten and Wysolmerski, 2003).

In humans, there are difficulties with sample collection, preparation and assays available for circulating PTHrP in pregnancy. Firstly, PTHrP quickly cleaves off and is easily degraded in serum. Suitable samples for PTHrP measurement are collected as EDTA plasma in a chilled tube which contains a protease inhibitor (such as aprotinin), and kept chilled on ice when being transported to the laboratory, it is spun within 15 minutes in a refrigerated centrifuge, separated, and frozen. Even with these measures to avoid proteolytic cleavage, PTHrP could begin to degrade in EDTA-aprotinin plasma within 15 minutes of sample collection.

When the PTHrP is not properly collected and processed, it leads to a failure in detecting the correct concentration and could lead to undetectable results when elevated values of PTHrP may be present (for example, in lactation or humoral hypercalcaemia of malignancy). This could lead to misinterpretation of results, since

majority of the assays measure $PTHrP^{1–86}$, and an undetectable result obtained (even though there is likely presence of PTHrP in the patient's circulation) may be misleading (Kovacs, 2016). Also, the most extensively used assays in clinical investigations are those that measure $PTHrP^{1–86}$ which does not exist in humans (or animals) (Kovacs, 2014). There are some mid-molecular and COOH-terminal forms of biologically active PTHrP. Present in humans are: $PTHrP^{38–94}$, $PTHrP^{38–101}$, $PTHrP^{107–139}$, and perhaps $PTHrP^{141–173}$, all of which may possess its receptor and distinct function (Kovacs, 2014). There is no data that is obtainable on concentrations of mid-molecular or COOH-terminal PTHrP in the course of pregnancy.

Even with the difficulties of PTHrP1–86 assay, some longitudinal studies have shown a significant rise in PTHrP throughout pregnancy, starting from the third week of gestation and attaining up to three times the first trimester value at term (Yadav *et al*., 2014). Another cross sectional study reported that PTHrP concentration in women at term was twice that of non-pregnant women (Glerean *et al.,* 2010). Some studies reported raised levels at term based on the normal values in non-pregnant women; it did not take into account a non-pregnant control group (Briana *et al*., 2007). These findings differ with smaller cross-sectional studies that reported $PTHrP^{1–34}$ or $PTHrP^{1–86}$ values were not different from unrelated non-pregnant controls or the expected normal range; also two longitudinal studies that discovered a non-significant rise and no variation in $PTHrP^{1–86}$ (Black *et al.,* 2000).

In the second study, all $PTHrP^{1–86}$ concentrations were undetectable, causing one to believe that the methodology was flawed (sample type, collection, and processing were not stated). On the postulation that the four longitudinal studies are accurate in

exhibiting a gestational increase in PTHrP that attains peak concentrations at term, PTHrP may significantly impact the control of mineral homeostasis in the mother during pregnancy, incorporating an upregulation in calcitriol (Yadav *et al.*, 2014). The tissue sources which play a part in the rising levels of PTHrP in the maternal circulation in human pregnancy has not been ascertained. Some of these sites may include the placenta, breasts, parathyroids, amnion, decidua and myometrium, umbilical cord, and other foetal tissues (Farrugia *et al*., 2000).

The possible significance of placental sources of PTHrP has been demonstrated by some clinical cases. A woman suffered a late-term hypercalcaemic crisis with increased plasma concentration of PTHrP; she was immediately C-sectioned and she had symptomatic hypocalcaemia within 6 hours with an undetectable PTHrP level (Eller-Vainicher *et al*., 2012).

Another woman with hypoparathyroidism was capable of gradually eliminating the use of calcitriol and reducing the dose of calcium to half taken during her pregnancy. Shortly after delivery, she acquired unexpected and symptomatic hypocalcaemia that ended after lactation started (Sweeney *et al.*, 2010). In the two cases, elimination of placental PTHrP likely caused an instant fall in serum calcium, while in the first case an instant and noticeable fall in circulating PTHrP was established. Some clinical cases have also proven that the breasts are a physiologically important source of PTHrP in the course of pregnancy, and can cause hypercalcaemia when PTHrP production is in excess.

This type of hypercalcaemia has increased in late pregnancy and the puerperium, and even in non-pregnant women who merely possess big breasts (Sato, 2008). Pregnant women have been shown to have PTHrP in their colostrum and milk, with the amount in colostrum not differing from that of breastfeeding women, while the PTHrP content increases in milk during each breastfeeding episode (Lubetzky *et al*., 2010). The steady increase in PTHrP across the trimesters, as established in four longitudinal studies, matches a gradual rise in serum calcitriol to peak levels in the third trimester (Yadav *et al.*, 2014).

The increase in PTHrP may indicate that it is accountable for stimulating renal 1α-hydroxylase (Cyp27b1). Though, other studies propose that PTHrP may not be as potent as PTH in stimulating Cyp27b1 *in vivo* (Wittelsberger and Rosenblatt, 2008). PTHrP accesses the maternal circulation during pregnancy, probably from the placenta and breasts, as well as possibly the uterus and foetal tissues. $PTHrP^{1–86}$ gradually rises in maternal plasma to attain peak levels in the third trimester, and is a possible factor in the normal control of mineral homeostasis during pregnancy. This comprises maintenance of the ionized calcium and stimulation of Cyp27b1 to bring about raised levels of calcitriol and suppression of PTH (Kovacs, 2016).

2.6 Calcitonin

Calcitonin was first discovered by Copp *et al.* in 1962, when they perfused isolated thyroid – parathyroid gland preparations from an anasthaesised dog. It was initially thought that the source of calcitonin was the parathyroid gland, but it was later estabilished by Pearse *et al* in 1966 that it was a a 32 amino acid polypeptide hormone that originated from the C – cells of the thyroid gland. While Potts *et al*. in 1992

determined the amino acid sequence of human and salmon calcitonin, which eventually led to the synthesis and commercial use of the more potent salmon calcitonin.

Salmon calcitonin, which differs from human calcitonin in 16 amino acids, has been used for treatment of hypercalcaemia. The hormone was first called thyrocalcitonin, but the name was eventually changed to calcitonin. Since its discovery more than 50 years ago, little progress has been made in understanding its pathophysiologic role in humans, in part because a deficiency or excess of calcitonin does not result in abnormalities except for diarrhea in a few patients with medullary thyroid carcinoma. Calcitonin decreases calcium and phosphate levels in the blood (Davey and Findlay, 2013). Its effects are opposite to that of the parathyroid hormone (Guyton and Hall, 2011).

2.6.1 Reference ranges for calcitonin

The reference ranges depend on the method used for assessment. The reference intervals based on sex and thyroid status that have been established in individuals are as follows:

Basal reference ranges for some calcitonin chemiluminescent assays: Less than 8.8 pg/mL (ng/L) in males; less than 5.8 pg/mL (ng/L) in females; less than 0.5 pg/ml (ng/L) in athyroidal individuals.

Peak calcium infusion (IMMULITE 2000 calcitonin assay): Less than or equal to 130 pg/mL in males; less than or equal to 90 pg/mL in females. Gender and age differences

in calcitonin have been shown with women having lower values than men and with decreasing calcitonin values with age in some, but not all studies have confirmed this trend (Deftos *et al*., 1980; Tiegs *et al*., 1986).

Pregnancy, lactation, and food intake have been known to influence calcitonin concentration in healthy subjects, but the specific reference ranges have not been established (Sofronescu, 2015). In this study we attempted to provide reference values for pregnant and lactating women in Zaria, Nigeria.

2.6.2 The physiology of calcitonin

In a review by Felsenfeld and Levine (2015) they discussed that calcitonin is primarily metabolized by the kidney, and the gene for calcitonin is on the short arm of chromosome 11 and encodes calcitonin and calcitonin gene-related peptide (CGRP). Calcitonin is found to be surplus only in the C-cells of the thyroid, while CGRP, which is a potent vasodilator, is not only present in the thyroid gland, but can also be found in both the central and peripheral nervous system.

Calcitonin is present in salt water fish because of the high concentration of calcium in their natural habitat with the need to extrude calcium out. Calcitonin is therefore older than parathyroid hormone (PTH) in discovery which was first discovered in early terrestrial animals when conservation rather than expulsion of calcium became pertinent. Asides regulating serum calcium and 1,25D production, other potential roles for calcitonin have been suggested and significant observations about calcitonin have been rendered. In uraemic and non-uraemic animal models, calcitonin has been shown

to reduce the extent of hypercalcaemia during calcium loading (Copp *et* al., 1962; Rodriguez *et al*., 1991).

Studies in the 1980s revealed that calcitonin enhanced renal production of Vitamin D (1,25-dihydroxycholecalciferol; 1,25D), (Rodriguez *et al*.,1991). However, in contrast to other stimuli of 1,25D production such as parathyroid hormone (PTH) and hypophosphataemia in which 1,25D production takes place in the convoluted proximal tubule, stimulation of 1,25D by calcitonin takes place inside the straight proximal tubule (Kawashima *et al.*, 1981). Similar to the mutual interactions involving both PTH, 1,25D, FGF23 and 1,25D, calcitonin stimulates 1,25D secretion, while 1,25D suppresses calcitonin secretion (Naveh-Many and Silver, 1988; Naveh-Many *et al.*, 1992).

However, during pregnancy and lactation, both 1,25D and calcitonin levels are increased (Kovacs and Kronenberg, 1997). The efficacy of calcitonin in the management of hypercalcaemia is as a result of reducing osteoclast activity and ensuring a break from the calcium-lowering effect of calcitonin (Heersche, 1992). Apart from reducing osteoclast activity, calcitonin has been proposed to expedite deposition of calcium and phosphorus in bone remarkably in the post-prandial state; but, whether calcitonin is stimulated in the course of food ingestion through gastrin stimulation and as a result, has an effect on bone modification has been a subject matter of interest (Talmage *et al*., 1980).

However, no differences in bone mass has been shown when calcitonin is absent in congenital hypothyroidism as long as thyroid hormone is replaced (Baran and

Braverman, 1991). Calcitonin screening has also been revealed to be a valuable instrument to help in diagnosing medullary thyroid carcinoma (Costante *et al*., 2007).

2.6.3 The role of calcitonin in hypercalcaemia

In a study using PTH knockout mice, PTH and calcium sensing receptor (CaSR) double knockout and wild mice, it was revealed that both CaSR-mediated calcitonin secretion and augmented renal calcium excretion were crucial for averting the occurrence of hypercalcaemia although the inhibition of PTH secretion was not a prerequisite for a strong defense against hypercalcaemia (Kantham *et al*., 2009). It can then be said that there is compelling evidence to indicate that calcitonin is a vital modifier of the hypercalcaemic effect of acute calcium loading (Felsenfeld and Levine, 2015).

Similar to PTH, calcitonin secretion is modified by the CaSR, although, when activation of the CaSR suppresses PTH secretion, calcitonin secretion is stimulated.

The mechanisms for the reverse effects downstream from the CaSR are still not properly comprehended (Felsenfeld and Levine, 2015). As with PTH, calcitonin gene transcription is suppressed by 1,25D (Naveh – Many and Silver, 1988), and the calcitonin and PTH receptors belong to the class II subclass of G-protein-coupled receptors (Davey and Findley, 2013).

2.6.4 Calcitonin and gender/age differences

A number of studies have reported that there are gender and age variations in serum calcitonin values. Deftos *et al* in 1980 stated that in normal adult subjects, basal calcitonin values were alike in men and women, but basal CT quantities declined with

advancement in age in both groups and the response to calcium changes was less in women than in men. Another study reported lower basal and stimulated calcitonin values in women than in men, but no decrease in calcitonin levels with age (Tiegs *et al.*, 1986). The calcitonin variation found between men and women has been attributed to a lower level of calcitonin secretion in women (Tiegs *et al.*, 1986).

In post-menopausal women, estrogen administration may raise calcitonin levels (Stevenson *et al.*, 1981) while, infants and children may also maintain greater serum calcitonin levels than adults (Body *et al.*, 1993). In a study by Silva *et al.*, (1981), it was reported that serum calcitonin levels increased towards the end of the third trimester and after delivery but it did not correlate with calcium or phosphate except during the first trimester where there was negative correlation with serum phosphate. They also suggested that the hypercalcitonaemia observed in pregnancy serves a protective function to the maternal skeleton, while the foetus accumulates calcium.

2.6.5 Calcitonin physiology in pregnancy and lactation

Due to the requirement of the foetus/infant for the transfer of maternal calcium, pregnancy and lactation are unique. Serum calcitonin and 1,25D levels are elevated during this period (Woodrow *et al.*, 2006). Qing *et al* in 2012 investigated the mechanism of calcium removal from bone during the period of lactation and they discovered that this is further promoted by a process called osteocytic osteolysis (Atkins and Findlay, 2012), whereas osteocytes remove the bone matrix surrounding them. Osteocyte expression of the PTH receptors appeared to be essential for lactation-induced osteocytic osteolysis since its removal tempered the decrease in Bone Mineral

Density (BMD) that ensues in lactation and inhibited the proliferation of osteocyte lacunae area observed in wild-type animals (Qing *et al.,* 2012).

Since CTR (calcitonin receptor) has been detected in osteocytes, it can be postulated that calcitonin may work at the level of the osteocyte to modulate osteocyte-mediated calcium release. In the past, the action of calcitonin to shield the maternal skeleton through lactation was believed to be facilitated only through restriction of osteoclast resorption (Davey and Findley, 2013). A breast, brain, and bone circuit in the control of mineral and skeletal metabolism during the reproductive cycle has been postulated as shown in figure 2.5 where calcitonin is manufactured in significant quantities in the mammary gland and in the pituitary gland, it may also have paracrine functions in the control of prolactin secretion (Kovacs, 2016). However, in what specific ways does calcitonin interact with these processes, remains unclear.

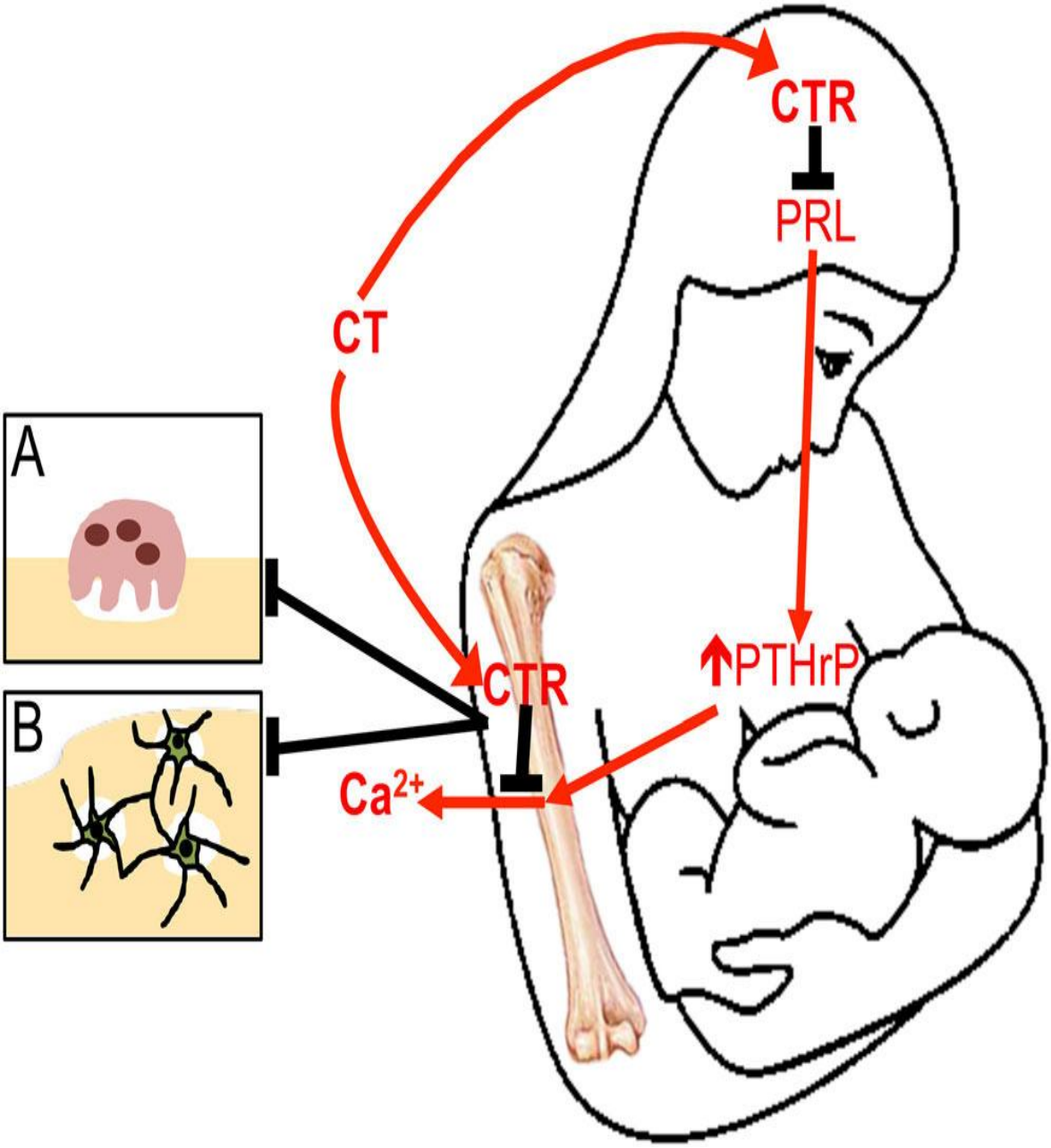


Figure 2.4: Potential actions of calcitonin (CT) to protect the skeleton during lactation

(i) Circulating CT derived from the thyroid and mammary tissue act via the calcitonin receptor (CTR) in the pituitary to decrease prolactin secretion, which in turn decreases the production of PTHrP by the breast; (ii) CT acting via the CTR to oppose the actions of PTHrP by inhibiting (a) osteoclastic bone resorption and (b) perilacunar/canalicular remodeling by osteocytes, in order to limit the amount of calcium released from the bone matrix (Kovacs, 2016).

2.6.6 Calcitonin and bone formation

There has been significant progress using genetically modified mouse models to investigate physiological functions for calcitonin acting through its receptor, the calcitonin receptor (CTR). The data that has been obtained from these studies has informed the suggestion that calcitonin has significant and associated functions in protecting the skeleton by controlling bone turnover and maintaining calcium homeostasis.

Based on the finding of calcitonin receptors on osteocytes, it has been hypothesized that calcitonin could potentially modify osteocyte products such as Fibroblast growth factor 23 (FGF23) or perhaps more importantly, sclerostin, a known regulator of bone formation. The detection of calcitonin receptors on osteocytes alludes to the fact that calcitonin may also modify the osteocyte's role (Davey and Finley, 2013). The ability of calcitonin to decrease serum calcium levels depends on the rate of bone turnover, (see figure 2.6). Atkins and Findley, 2012 discussed the pool of exchangeable calcium that is in equilibrium with bone, the efflux of which appears to be influenced by PTH, with the influx perhaps taking place along a physicochemical gradient. However, it is not known if calcitonin influences the latter, but a greater effect on calcium efflux in conditions of high bone turnover has been found to be consistent with its inhibitory action on osteoclasts, and possibly osteocytes (Gooi *et al.*, 2010; *Qing et al.*, 2012).

The capability of calcitonin to lower serum calcium after an exogenous calcium load can be partially accounted for by decreasing the efflux of calcium from bone, instead of modifying its influx. In fact, Calcitonin/ Calcitonin gene related peptide knock out (CT/CGRPKO) mice similarly exhibited a pronounced calcaemic response to

exogenous PTH administration than controls as a result of enhanced bone resorption, which was stopped by treatment with calcitonin but not Calcitonin Gene Related Peptide (CGRP) (Hoff *et al.*, 2002). Data from studies in global-CTRKO and osteoclast-CTRKO mice have demonstrated that the effect of calcitonin to protect the body from hypercalcaemia is basically facilitated through the CTR on osteoclasts. Hypercalcaemia caused by treatment with $1,25(OH)_2D$ causes a higher hypercalcaemic reaction in global-CTRKO and osteoclast-CTRKO mice when compared to littermate controls; this was attributed to escalated bone resorption.(Davey *et al.*, 2008; Turner *et al.*, 2011).

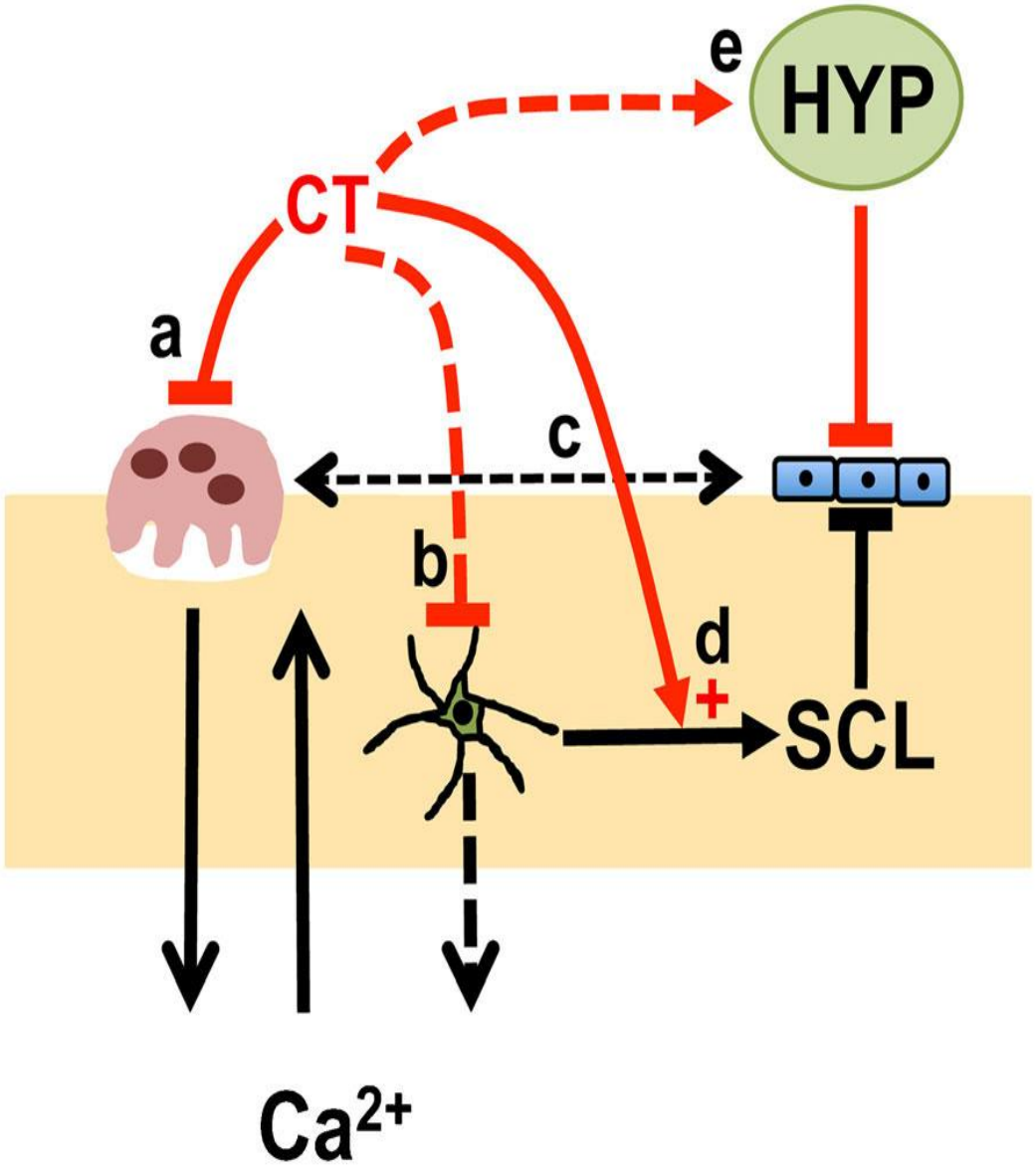


Figure 2.5: Proposed model by which calcitonin (CT) protects the skeleton by regulating bone turnover.

These actions appear to be most important when bone turnover is high. CT limits calcium flux from bone by (a) inhibiting osteoclastic bone resorption and (b) perilacunar/canalicular remodeling by osteocytes. Calcitonin may act to (c) regulate coupling between osteoclasts and osteoblasts; inhibit bone formation by (d) stimulating the production of sclerostin fromosteocytes; and (e) by acting via the CTR in the arcuate nucleus of the hypothalamus to activate a yet unidentified efferent neural pathway. Pathways indicated by dashed lines represent hypothesized mechanisms of CT action requiring further investigation. (Davey and Findley, 2013)

2.6.7 Calcitonin disorders and deficiency in pregnancy

There are extrathyroid sites of calcitonin synthesis in the pregnant state such as the mammary glands and the placenta (Kovacs, 2016), and as such calcitonin deficiency may not be completely evident even when complete thyroparathyroidectomy is performed on test subjects. However some studies using animal models (goats and rats) have attempted to induce calcitonin deficiency by a complete thyroparathyroidectomy followed by parathyroid gland autotransplantation and treated with exogenous thyroid hormone (Chalon and Garel, 1985). T, serum calcitonin, TSH, PTH, and ionized calcium were not quantified, so it is not known if a calcitonin-deficient, euthyroid, euparathyroid status was attained (Kovacs, 2016). In these studies, the ash weight and calcium content were 5–10 % lower in the thyroidectomized when compared with sham operated animals at the end of pregnancy. The differences were considered statistically significant in goat metacarpals and metatarsals, but not in rat femora (Chalon and Garel, 1985).

Some human studies have suggested that increased serum calcitonin protects the pregnant woman's skeleton from extreme resorption during pregnancy, but this has not been verified. Some completely thyroidectomized pregnant women still produce calcitonin from the placenta and mammary glands and as such are not truly calcitonin deficient. Women with genetic absence of calcitonin or its receptor have not been studied during pregnancy.

Yet, a number of reports have examined the long-term consequences of total thyroidectomy on bone density and the danger of fractures in men and women. A number of reports have proposed an increased risk, while others have concluded none.

In the last few years, investigations have been puzzled by the usual practice to treat with suppressive doses of thyroid hormone to decrease the risk of cancer relapse, however, even those studies have not found bone loss to be consistent (Kim *et al.*, 2015; Lee *et al.*, 2014). Not one of these studies has investigated the effects of pregnancy, of which the women may not even have calcitonin deficiency.

It is remarkable that with recent calcitonin assays, the basal calcitonin level is unaffected before and after total thyroidectomy in patients who do not have medullary thyroid cancer. Only the calcium-stimulated or pentagastrin-stimulated calcitonin is lost in these patients (Kudo *et al.*, 2011). This implies that even in non-pregnant, non-lactating women, extrathyroidal calcitonin sources are functional. Thus, it can be said that thyroidectomy to cause partial calcitonin deficiency during pregnancy leads to bone loss at term compared with shamoperated animals; though, no effect was observed in pregnant mice deficient in the calcitonin gene. No studies with human data can be compared for the surgical or genetic models of calcitonin deficiency during pregnancy.

2.7 Skeletal Turnover and Mineral Metabolism

Intestinal calcium absorption is increased during the first trimester, this is long before the foetal demand for mineral in the third trimester. Women are usually in a positive calcium balance by mid-pregnancy as shown in some metabolic studies, so skeletal mineral content may become increased at that time-point to justify where that calcium is stored. Though, the absence of data on bone mass, structure, or mineralization during mid-pregnancy makes it difficult to conclude. Some maternal bone resorption during late pregnancy may contribute mineral to the foetus. This has been demonstrated by a

study of women who lived near the Techa River in Russia, who unintentionally ingested Strontium-90 (^{90}Sr) from water contaminated by nuclear waste in the 1950s. Long after the exposure was over, ^{90}Sr could still be detected in the skeletons of their foetuses, which could only have come from resorption of ^{90}Sr that had been previously fixed in the maternal skeleton (Tolstykh *et al.,* 2001). Similarly, women who ingested ^{90}Sr during pregnancy, particularly during the third trimester, gave birth to babies with 10-fold higher ^{90}Sr content, consistent with dietary absorption of mineral being accountable for much more of the mineral content of the foetal skeleton (Shagina *et al*., 2007; Tolstykh *et al.,* 2001).

The only study reporting iliac crest histomorphometry during human pregnancy was done in 15 women who were scheduled to have elective first trimester abortions, 13 women scheduled to have C-sections in the third trimester, and 40 non-pregnant women (some living and some cadaveric specimens). At the first trimester time point, bone resorption indexes were increased, bone formation indexes were decreased, and trabecular bone volume was significantly decreased, when compared with non-pregnant women.

Conversely, the third-trimester biopsies were no different from the non-pregnant values for bone mass and indexes of bone resorption and formation. Seen at a glance, these results allude that early pregnancy is a bone-resorptive state, which brings about bone loss that is later regained by the end of pregnancy. This explanation does not fit easily with the fact that little transfer of calcium is taking place from mother to offspring during the first trimester; as a consequence of which, skeletal resorption should not be increased at this time point, especially given that intestinal calcium absorption has been

upregulated. Rather, increased bone resorption has a tendency to occur in the third trimester when the foetal demand for mineral is at its peak. The detected differences may be the result of interaction between small sample sizes and other confounding factors among the groups. Age matching was poor and may have been a factor for the apparent differences, with mean ages of 22.4 years in the first trimester group, 25.1 years in the late pregnancy group, and 32.6 years in the non-pregnant group. More studies on histomorphometric data from pregnant women are required (Kovacs, 2016).

Cross-sectional and serial measurement of bone turnover markers have been done in pregnant women. These indicators are generally considered more accurate at detecting changes in bone resorption when compared with bone formation and are subject to diurnal variation as well as differences between fasted and postprandial values (Lombardi *et al.,* 2012). Pregnant women were often compared with non-pregnant controls or normal ranges, rather than pre-pregnancy measurements from the same women. Additional potential confounders include haemodilution which affects serum measurements (confirmed to affect osteocalcin and serum collagen type 1 cross-linked C-telopeptide [CTX], Kaur *et al.,* 2003), increased glomerular fileration rate (GFR) and altered creatinine excretion affecting urinary measurements; possible contributions from placenta and other tissues, increased degradation or clearance by the placenta (confirmed to affect osteocalcin), and lack of fasted or diurnally timed specimens.

Overall, available data suggest that bone turnover increases during pregnancy, especially during the third trimester. There are real but small declines in bone mineral density throughout the skeleton that require a sufficiently large cohort to be detected with confidence. Long-term impacts of skeletal changes induced by pregnancy has

been examined through numerous epidemiological studies in order to ascertain whether parity confers any risk of lower BMD, an osteoporotic level of BMD, or fragility fractures. The majority of such studies have found either a neutral effect of parity (Heidari *et al.*, 2013; Mori *et al.*, 2015) or that parity confers greater BMD (Paton *et al.*, 2003; Schnatz *et al.*, 2010). Other studies have reported that parity decreases the risk of hip fractures (Kauppi *et al.*, 2011).

One of the studies reported a protective effect of parity was particularly strong because it involved 1,852 twins and their female relatives, including 83 identical twins that were discordant for ever being pregnant (Paton *et al.*, 2003). For balance, a few studies have found that parity is associated with reduced BMD (Khoo *et al.*, 2011; Heidari *et al.*, 2015) or increased vertebral (Bolzetta *et al.*, 2014) or hip fracture risk. However, these reports are substantially more than the aforementioned studies that found a neutral or protective effect of parity. There are also divergent effects among the different studies. For example, one study reported that parity reduces femoral neck BMD but had no effect on lumbar spine. Another study found that parity reduces total hip and femoral neck BMD in postmenopausal women, but not in premenopausal women who are closer in time to the event and therefore less likely to show confounding effects (Zhang *et al.*, 2003).

Available data from pregnant women indicate that a positive calcium balance also occurs by mid-pregnancy, but whether BMD is objectively increased at this time point has not been determined. Upregulation of bone resorption occurs in the third trimester and likely causes some bone loss, with most women ending up with either no change or a very modest decrease in BMD by term. Increased resorption and net bone loss are

more likely to occur in women who do not absorb sufficient calcium to meet the combined needs of themselves and their babies. Inadequate dietary calcium intake probably contributes to increased risk of pregnancy-associated osteoporosis. In the long term, these pregnancy-related changes in bone metabolism or bone mass appear to have no adverse effect on the calcium content or strength of the maternal skeleton, and may even have a protective effect against low BMD and future risk of fractures (Kovacs, 2016).

2.8 Disorders of Bone and Mineral Metabolism during Pregnancy

Osteoporotic fractures during pregnancy in humans are very uncommon, but they do occur. They have probably been occurring in association with reproduction for the past five millennia, because vertebral compression fractures and low BMD have been found in Egyptian mummies and other archaeological skeletons of women who were 16–30 years of age when they died (Shilbayeh, 2003). Osteoporosis may present in two ways: vertebral fractures and so-called transient osteoporosis of the hip.

2.9 Calcium Intake, Vertebral and other Fractures in Pregnancy.

Fragility fractures of the spine (and less commonly other skeletal sites) have occurred during the third trimester, and in the puerperium of women who did not breastfeed. In both situations these fractures may be considered to have been provoked by pregnancy. In most affected women there is no preceding bone mineral density (BMD) reading because of no prior indication for it to have been done. Serum chemistries and calciotropic hormone levels are usually unremarkable. However, bone loss is likely a factor in pregnancy-related vertebral fractures, because the BMD is usually low at presentation (Philips *et al*., 2000), and it spontaneously improves afterward (Philips *et*

al., 2000; Iwamoto *et al.*, 2012). In the few cases where bone biopsies were done, mild osteoporosis was observed. Insufficient calcium absorption (from dietary calcium deficiency, lactose intolerance, celiac disease, other malabsorptive disorders, and vitamin D deficiency) is likely to induce resorption of the maternal skeleton during the third trimester. A recent report described a woman with a calcium intake of 229 mg daily, who developed multiple vertebral compression fractures during pregnancy (Kovacs, 2015). Since that amount was insufficient for her own non-pregnant needs, the normal upregulation of intestinal calcium absorption during pregnancy would not have benefited her.

Significant skeletal resorption was an inevitable consequence of her pregnancy. Another potential cause of physiological bone loss during pregnancy is greater than normal release of PTHrP from the placenta and breasts. The gradual rise in plasma PTHrP likely contributes to upregulation of calcitriol synthesis, intestinal calcium absorption, and bone turnover in all pregnant women. However, on individual basis, high circulating levels of PTHrP have arisen from the breasts or placenta and led to pseudohyperparathyroidism, which is characterized by increased bone resorption and severe hypercalcaemia (Eller-Vainicher, 2012).

High circulating levels of PTHrP that contributed to bone resorption have also been noted in women who presented with fragility fractures weeks after delivery. Such high levels may have begun in pregnancy and contributed to bone loss before delivery (Anai *et al*., 1999). Additional factors that contribute to fracture risk during pregnancy include increased weight-bearing (average weight gain of 12 kg), lordotic posture, reduced bone mass or strength that precedes pregnancy, and conditions that cause bone

loss during pregnancy. Published cases have revealed such diverse conditions as anorexia, longstanding hypomenorrhea or relative estradiol deficiency, premature ovarian failure, petite stature or body frame, mild osteogenesis imperfecta, inactivating mutations in lipoprotein receptor-related protein 5 (LRP5), dietary calcium deficiency, renal calcium leak (hypercalciuria), bedrest and inactivity, and pharmacotherapy before or during pregnancy that may induce bone loss (heparin, oral glucocorticoids, gonadotropin releasing hormone [GnRH] analogs, depot medroxyprogesterone acetate, and certain anticonvulsants) (Kovacs, 2015).

A maternal family history of severe osteoporosis is likely to be present in women with pregnancy-associated fragility fractures, which implies that genetic causes of low bone mass or skeletal fragility may be present. Pregnancy-associated osteoporosis typically occurs in a first pregnancy, higher parity does not increase the risk, and fractures usually do not recur in subsequent pregnancies (Philips *et al.,* 2000). These observations imply that in many cases reversible factors (such as nutritional deficiencies) may have been corrected after the first pregnancy. Permanent disorders such as osteogenesis imperfecta or LRP5 mutations should be anticipated to maintain increased risk of vertebral and other fractures in subsequent pregnancies.

Many uncontrolled treatments such as nasal calcitonin, bisphosphonates, strontium ranelate, and teriparatide (Ozturk *et al.*, 2014; Campos-Obando *et al.,* 2014; Zarattini *et al.,* 2014; Dytfeld and Horst-Sikorska, 2012) have been reported in various case series. Most of the authors of these reports evidently did not appreciate that substantial spontaneous recovery of bone mass occurs even in women who fractured, with BMD increases in the range of 20–70% in individual cases. For example, a 50% increase in

BMD was demonstrated by quantitative computed tomography (QCT) in a woman who presented with a compression fracture 1 week postpartum. Therefore, the attributable effect of pharmacological therapy may have been greatly overestimated. It may be reasonable to wait 12–18 months for spontaneous recovery to occur before evaluating whether the fracture risk remains high enough to warrant pharmacological therapy (Kovacs, 2015).

2.10 Disorders of Parathyroid Hormone Secretion

2.10.1 Primary hyperparathyroidism

The exact prevalence of primary hyperparathyroidism during pregnancy is uncertain. A recent retrospective series found that it occurred in 0.03% of reproductive age women who underwent routine screening of serum calcium (Cabiddu *et al.,* 2015). Several hundred cases have been described by Cabiddu *et al.,* 2015, while their advice has been sought for several cases each year by clinicians around the globe. Primary hyperparathyroidism is 10–20 times more likely to occur in women over age 45 than in women of child-bearing age, but in two case series, ~ 1% of all parathyroidectomies were done in pregnant women. The pathology is similar to non-pregnant cases: a predominance of single adenomas followed by four-gland hyperplasia (El-Hajj Fuleihan and Arnold, 2013).

The physiology of normal pregnancy can worsen hypercalcaemia caused by primary hyperparathyroidism since both conditions cause increased intestinal calcium absorption, hypercalciuria, and some degree of increased bone resorption. This may explain the perceived increased risks of severe hypercalcaemia, pancreatitis, and kidney stones when primary hyperparathyroidism occurs during pregnancy. Conversely, rapid placental calcium transfer and uptake of calcium by the foetal

skeleton during the third trimester may help protect against severe hypercalcaemia in the mothers. Loss of outflow to the placenta likely explains why hypercalcaemic crises have occurred after delivery of the placenta. Physical inactivity and bedrest will add an additional component of skeletal resorption (Nilsson *et al.*, 2010; Paul *et al*., 2012).

2.10.2 Hypoparathyroidism

Hypoparathyroidism is usually known to be present prior to pregnancy. However, in specific cases, asymptomatic women have not been recognized until the newborn presented with severe secondary hyperparathyroidism, hypercalcaemia, increased bone resorption, and fractures (Demirel *et al.*, 2009). If the mother remains hypocalcaemic during pregnancy, maternal hypoparathyroidism can have a serious adverse impact on the foetus and neonate (Kovacs, 2014). Animal data indicates that hypoparathyroidism can improve or worsen during pregnancy, and the same divergent outcomes are true for hypoparathyroidism in women (Kovacs, 2014).

2.11 Vitamin D Deficiency and Genetic Disorders of Vitamin D Physiology

Calcitriol regulates the active, saturable, transcellular mechanism of intestinal calcium delivery, as well as the passive, paracellular mechanism of absorption (Christakos, 2012). As in rodents, calcium represents ~ 20% of net calcium absorption, with the rest occurring by passive, non-saturable mechanisms. Active absorption is considered to be more important when dietary intake of calcium is limited. Multiple dual or single isotope studies carried out in adults and adolescents have examined the level of 25OHD at which intestinal calcium absorption is maximized or reaches a plateau, and have established that a plateau occurs below 20 nM 25OHD, with very little increase (if any) in intestinal calcium absorption above that value (Aloia *et al,* 2014; Gallagher

et al., 2012). Secondary hyperparathyroidism occurs at lower levels of 25OHD and maintains high levels of calcitriol despite the reduced availability of substrate, which may be why vitamin D deficiency does not impair intestinal calcium absorption until the 25OHD level is below 20 nM.

There is no evidence to support that maternal vitamin D deficiency, or genetic disorders causing loss of calcitriol or Vitamin D Receptor (VDR), affect maternal mineral metabolism and obstetrical or foetal outcomes. Because no study has measured intestinal calcium absorption during pregnancy in vitamin D-deficient compared with vitamin D-sufficient women, it remains unknown whether or not intestinal calcium absorption doubles in pregnant, vitamin D-deficient women (Kovacs, 2014). The foetal and neonatal outcomes of these studies have been described, and no consistent change in cord blood calcium, phosphorus, PTH, birth weight, or anthropometric measurements were observed when babies of vitamin D-supplemented mothers were compared with babies of placebo-treated mothers.

Several studies did note a reduction in the incidence of neonatal hypocalcaemia after 48 h when the babies of placebo-treated mothers had cord blood 25OHD levels below 20 nM (Kovacs. 2014). This is consistent with the aforementioned adult data that intestinal calcium absorption is reduced when the 25OHD level is below 20 nM. However, calcium absorption across the foetal intestines is a trivial circuit not relevant to foetal mineral homeostasis, whereas the main route of calcium delivery is transportation across the placenta through mechanisms that do not require calcitriol (Kovacs, 2014). Hollis and Wagner, 2011 reported on 350 women (NCT00292591) randomized at 12–16 weeks of gestation to receive 400, 2,000, or 4,000 IU vitamin D_3

per day. Maternal 25OHD levels at term were 79, 98, and 111 nM (31.6, 39.2, and 44.4 ng/ml), respectively. There was no significant difference in serum calcium across the treatment groups, but there was a borderline-significant reduction in PTH when the high-dose and low-dose groups were compared. There were no differences in any obstetrical outcomes, including mode of delivery, gestational age at delivery, preterm birth, preterm labour, pre-eclampsia, and infection (Hollis and Wagner, 2011).

Dawodu *et al*., 2013 studied Arab women who had mean 25OHD concentrations of 20.5 nM (8.2 ng/ml) at baseline and were randomized to 400, 2,000, or 4,000 IU vitamin D/day. Achieved 25OHD levels at delivery were 40 nM (19.3 ng/ml), 65 nM (25.9 ng/ml), and 90 nM (35.9 ng/ml), respectively (Dawodu et al., 2013). There were no differences in maternal serum calcium or urine calcium/ creatinine between groups at any time point during pregnancy, PTH was reduced in the high-dose group but correlated poorly with 25OHD; no effect was seen on gestational age at delivery or the babies' anthropometric parameters at birth, and no other obstetrical outcomes were reported. Most recently, Cooper and Harvey and co-workers (Cooper *et al*., 2016) provided preliminary results of the Maternal Vitamin D Osteoporosis Study (MAVIDOS) study from the United Kingdom. The 900 women who completed the study randomly received either 1,000 IU vitamin D or placebo beginning at 14 weeks of pregnancy. Baseline 25OHD was ~45 nM (18 ng/ml), did not change in placebo-treated women, and rose to 68 nM (27 ng/ml) near term in the vitamin D-supplemented women.

During the oral and poster presentation of results (Cooper *et al*., 2016), the study was revealed to be negative in its primary outcome (neonatal bone area, bone mineral

content, and bone mineral density within the first 14 days after birth) and secondary outcome (anthropometric and body composition parameters within 48 h of birth). No obstetrical benefit was reported. On the basis of associational studies, vitamin D deficiency has been suggested to have non-skeletal effects during pregnancy, such as increasing the risk of preterm delivery, C-sections, low birth weight, preeclampsia/pregnancy-induced hypertension, and vaginal infections. One can find approximately as many studies suggesting these associations (Bodnar *et al.,* 2007; Shibata *et al.,* 2011) as there are studies indicating no association (Powe *et al.,* 2010; Shand *et al.,* 2010). Each of these studies has low power and are confounded by factors that may lead to lower 25OHD levels and the outcome in question, including race/ethnicity, maternal overweight/obesity, lower socioeconomic status, poor nutrition, e.t.c.

2.12 Disorders of Bone and Mineral Metabolism during Lactation

Animal studies have shown that despite significant skeletal resorption during lactation that is most marked in the lumbar spine, it is unusual for fractures to be found. This is likely because the rodent ambulates on four limbs, thereby not loading the spine in the same orientation or to the same degree as in a bipedal human. While in humans, multiple case reports and series have confirmed that, although rare, osteoporotic fractures can occur in breastfeeding women (Kovacs, 2015). Consistent with most bone loss occurring in the axial spine, vertebral compression fractures have been reported, sometimes with as many as 6–10 such fractures evident at presentation in one individual (Bonacker *et al.,* 2014; Ozturk *et al.,* 2014).

These fractures can be quite debilitating. The literature is confusing because many cases are described as “pregnancy-associated osteoporosis” despite the fractures occurring several months into lactation. In some breastfeeding women, the physiological bone resorption induced by PTHrP and low estradiol may be a sufficient explanation for reduced skeletal strength that predisposes to fragility fractures. As noted earlier, in some individuals the lumbar spine BMD has been shown to drop from normal to osteoporotic values during otherwise normal lactation. In some instances release of PTHrP by the breasts may be more exuberant than normal, leading to more marked resorption of bone.

Three such breastfeeding women presented with hypercalcaemia, increased PTHrP, and vertebral compression fractures. The hypercalcaemia and increased PTHrP resolved in all three women after weaning but in one woman the plasma PTHrP level remained elevated for at least 4 months after lactation ceased. Three other cases of compression fractures during lactation also had hypercalcaemia that resolved after weaning, which is suspicious for elevated PTHrP as the cause. PTHrP was undetectable in one case and appropriate for lactation in two others, but common problems with sample collection and processing may explain why high levels of PTHrP were not found.

It is also conceivable that relative sensitivity to the skeletal-resorptive effects of PTHrP may differ among women, thereby contributing to differences in the amount of bone lost during lactation. On the other hand, review of the individual cases indicates that in many women there are additional factors that contribute to skeletal fragility, including causes of low bone mass or fragility that precede pregnancy, and bone loss that likely

occurs during pregnancy (such as with very low calcium intake). This leads to the consideration that if a woman is known to have skeletal fragility or very low bone mass prior to pregnancy or at delivery, it may be reasonable to advise against breastfeeding in case the programmed skeletal resorption proves to be too much for that woman's skeleton (Kovacs, 2015). When fractures do occur, it may also be reasonable to advise that breastfeeding cease to stop further bone loss and initiate the expected post-weaning skeletal recovery.

Individual cases have reported spontaneous improvements in bone density by 20–70% (Philips *et al.,* 2000; Iwamoto *et al.,* 2012); thus pharmacological therapy may be reasonably withheld for 12–18 months to determine the magnitude of spontaneous recovery (Kovacs, 2015). An anti-remodelling agent such as a bisphosphonate or denosumab (used to treat bone loss) might blunt the spontaneous bone formation that is expected during the post-weaning interval. As noted earlier, individual case reports have described use of nasal calcitonin (Dytfeld and Horst-Sikorska, 2012; Ozturk *et al*., 2014), bisphosphonates (Choe *et al.,* 2012; Campos-Obando *et al.,* 2014), strontium ranelate (Zarattini *et al.,* 2014), and teriparatide (Choe *et al.,* 2012; Lee *et al.,* 2013; Bonacker *et al.,* 2014) to treat osteoporosis diagnosed during pregnancy or lactation. In each of these uncontrolled cases, the achieved BMD increase was assumed to be due to pharmacological therapy, but remained within the expected range of increase that can occur spontaneously during post-weaning recovery.

Hence, we could summarise by saying that fragility fractures of the spine and other sites rarely occur during lactation and may result from the normal physiological resorption of the skeleton during lactation, combined with effects of low bone mass or

skeletal fragility that preceded pregnancy, and skeletal resorption that may have occurred during pregnancy (Kovacs, 2015). A spontaneous and substantial improvement in bone mass occurs after weaning even in women who have fractured, so pharmacological therapy should probably be withheld for at least a year to determine if it is really needed.

2.13 Oxidative Stress in Pregnancy

Under normal conditions in the human body, reactive oxygen species and antioxidants are maintained in a balance. The oxidative stress occurs when this balance is shifted towards an overabundance of reactive oxygen species. And oxidative stress results from an imbalance between pro-oxidants (free radical species) and the body's scavenging ability (antioxidants) (Suhail *et al.,* 2010). Pregnancy is associated with progressive and periodic changes in the metabolic and physiology of the body of the woman. Therefore, remarkable and dramatic events occur during this period for sustaining the mother and fostering the growth and maintenance of foetus.

Pregnancy though not a disease is associated with a high-energy demand of many bodily functions and an increased oxygen requirement (Gitto *et al.,* 2002). During pregnancy, minute ventilation increases by 36% at the 8th week of pregnancy. Pregnancy also has an effect on maternal antioxidant enzyme activities. Glutathione peroxidase and superoxide dismutase activities have been found to be reduced during the second trimester of pregnancy in humans. Such conditions may be responsible for raised oxidative stress in pregnancy.

Malondialdehyde (MDA) is a decomposition product of peroxidized polyunsaturated fatty acids, and is widely used in evaluating the degree of oxidative stress in various pathological conditions (Nair-Shalliker *et al.,* 2008). The production of malondialdehye and other aldehydes is used as a biomarker to measure the level of oxidative stress in an organism (Del Rio *et al.,* 2005).

Antioxidants are substances generated endogenously or taken as exogenous compounds with the ability to block the initiation of free radical formation or scavenge the free radical formed (Aurousseau *et al.,* 2006). Examples of such compounds with antioxidant properties are vitamins A, C, D and E, sulhydryl-containing compounds such as cysteine and glutathione and serum proteins, such as albumin, ceruloplasmin and transferrin and enzyme system such as superoxide dismutase.

The other relevant antioxidant systems include superoxide dismutase, albumin, glutathione and selenium. Some studies have shown that malondialdehyde levels were higher in pregnant mothers as compared to age matched non-pregnant women and at the same time antioxidant levels were found to be significantly low due to higher antioxidant requirement and intake, metabolic demand, hemodilution or a combination of these.

Recently, some studies have shown that Vitamin D supplementation during pregnancy might affect pregnancy outcomes through influencing insulin resistance, systemic inflammation, and oxidative stress by inducing superoxide dismutase (Zhang *et al*., 2009; Halicka *et al.,* 2012; Wagner *et al.,* 2013) In addition, improved levels of systemic inflammation as well as favourable pregnancy outcomes (Buppasiri *et al.,*

2011) were seen with taking calcium supplements during pregnancy. Among non-pregnant women, calcium supplementation has resulted in decreased insulin resistance. Despite this positive documentation, findings from a meta-analysis revealed that calcium supplementation without Vitamin D might increase the risk of cardiovascular events (Bolland *et al.*, 2011). Therefore, the role of calcium supplements in the management of chronic conditions, in particular during pregnancy, needs further assessment. A confounding study has however shown that vitamin D decreases the activity of superoxide dismutase (Lycans *et al.*, 2016).

Although some studies have shown that Vitamin D supplements alone had similar effects on serum Vitamin D levels, compared to combined calcium-vitamin D supplementation, others believe that the efficacy of a single supplementation with calcium or vitamin D would be improved with joint supplementation. Calcium-Vitamin D co-supplementation might affect inflammation, and oxidative stress through their effects on the regulation of cell cycle, activation of antioxidant enzymes, and inhibition of the synthesis of inflammatory cytokines (Hajsadeghi *et al.*, 2012; Nair-Shalliker *et al.*, 2012) These favourable effects might mediate the effect of the co-supplementation on pregnancy outcomes (Asemi *et al.*, 2016).

In a study by Asemi *et al.*, 2016, pregnant women were randomly allocated to consume either the placebo or calcium-Vitamin D supplements (containing 500 mg calcium carbonate plus 200 IU Vitamin D_3 or cholecalciferol) once daily for 9 weeks but in this study, the subjects were not given any supplements.

Glutathione peroxidase levels in pre-eclamptic patients were investigated recently by Roland-Zejly *et al*. (2011), suggesting that decreased GSH-Px activity and increased oxidative stress in placental villi may be attributed in part to GSH-Px 4 isoenzyme.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The equipment and materials used for the study were as follows:

3.1.1 Chemicals and reagents

Human vitamin D, Human Parathyroid hormone (PTH) and, Human Calcitonin (CT) ELISA kits were procured from Wkea Medical Supplies Corporation in Jilin, China, methylated spirit, distilled water, thiobarbituric acid, serum samples, trichloroacetic acid, ammonium molybdate, sulphuric acid 0.2M phosphate buffer (8.40g of NaH_2PO_4, 9.94g of Na_2HPO_4), 30mM hydrogen peroxide, (freshly prepared and kept in amber bottle), 0.05M carbonate buffer.

3.1.2 Equipment

Audicom auto-electrolytes analyzer (Model: AC9900)**,** microplate reader (Model 680) (capable of measuring absorbance at 450 nm), spectrophotometer, stop-watch.

3.1.3 Materials

Plain serum bottles, 10 ml syringes, cotton wool, methylated spirit, tourniquet, serum samples, single-channel automated pipette, precision pipettes (2 mL and 1 mL), multi-channel precision pipettes with disposable tips (10- 100 µL and 50- 200 µL), pipettes (adjustable 10 ml- 100 ml) for reagent preparation, measuring cylinders (100 ml and 1 liter), squirt bottle, absorbent paper, incubator (37^oC), filter paper, graph paper, test-tubes for standard and sample dilutions, measuring cylinder, beakers, cuvettes, centrifuge, water bath, stop-watch and volumetric flasks (1000mL, 100mL and 20mL).

3.2 Demography of Study Area

Zaria is a heterogeneous city in Kaduna State. It is inhabited by about 1,018,827 people (National Bureau of Statistics, 2015). Kaduna is located in the North-west geopolitical zone of Nigeria, Kaduna State is one of the 36 states in Nigeria with its capital in Kaduna city. Zaria occupies a portion of the high plains of Northern Nigeria, 652.6 meters above sea level and some 950 kilometers from the coast at11^{o}031N, 7^{o}42E. Zaria is home to several tertiary institutions of learning such as the Ahmadu Bello University (A.B.U.) Zaria, Nigerian College of Aviation Technology (NCAT), Nigerian Institute of Transport Technology (NITT), National Research Institute for Chemical Technology (NARICT), Federal College of Education (FCE) Zaria; just to mention a few. It is located within the Northern Guinea Savanna agro – ecological zone. Its annual rainfall ranges from 0.0 – 816.0mm/month. Minimum and maximum ambient temperature in Zaria averages 15.3^{o}C and 36.25^{o}C (Marthins, 2006), respectively.

3.3 Study Design and Population

This study is a cross sectional descriptive study, that was conducted between August and September 2015. A total of one hundred and seventy nine (179) women attending antenatal, family planning and immunisation clinics at the Ahmadu Bello University Teaching Hospital (ABUTH) Shika, Zaria, Maternal and Child Health Centre, ABUTH, Ban Zazzau, Ahmadu Bello University (ABU), Medical Centre and, Comprehensive Health Centre, ABUTH, Sabon Gari, Zaria were recruited for the study. A structured questionnaire about pregnancy history was administered. Blood samples were collected by a qualified Laboratory Scientist at the Departments of Obstetrics and Gynaecology, ABUTH, Shika and Comprehensive Health Centre,

ABUTH, Sabon Gari, Zaria. Anthropometric measurements (body weight and height) were also recorded from the subjects. Body mass Index (BMI) was calculated from data collected from the anthropometric indices. The subjects were classified based on ethnicity, occupation and residential area.

I. Classification based on ethnicity:

   i. Hausa

   ii. Yoruba

   ii. Igbo

   iii Others (all other ethnic groups apart from Hausa, Yoruba and Igbo)

II Classification based on occupation:

   i. Housewife: women who do not earn income

   ii. Handwork: women with semi-formal training who earn some income such as seamstresses, hairstylists, etc.

   iii Civil Servant: women with formal training who earn income in a formal setting.

III. Classification based on Residential Area:

   i. Ahmadu Bello University (A.B.U.): in and around Ahmadu Bello University, Zaria

   ii Tudun Wada (T/Wada): Tudun Wada, Zaria and its environs.

The women were grouped as follows: (n = 179)

Group 1: Non-pregnant women: Control group (n = 15)

Group 2: Pregnant women in the first trimester: 0 – 13 weeks (n = 40)

Group 3: Pregnant women in the second trimester: 14 – 26 weeks (n = 41)

Group 4: Pregnant women in the third trimester: 27 – 38 weeks (n = 53)

Group 5: Lactating women (n = 30)

3.4 Inclusion Criteria

Healthy consenting women attending antenatal, family planning and immunisation clinics at Ahmadu Bello University Teaching Hospital (ABUTH) Shika; Women and Child Centre, ABUTH, Ban Zazzau; Women Centre, ABUTH Sabon Gari, Antenatal Clinic, Sickbay, Ahmadu Bello University.

3.5 Exclusion Criteria

Pregnant women with any disorder that affects metabolism of calcium or bone, history of endocrine, renal or liver illnesses, hypertension of pregnancy, gestational diabetes, thyroid disorders, cancer or treatment with anti-tubercular or antiepileptic drugs in the previous 3 months were excluded from the study.

3.6 Sample Size Determination

The formula for determination of the sample size used was for comparing two means, as follows (Kirkwood and Sterne, 2003):

$$n = \frac{(u + v)^2 (O_1^2 + O_0^2)}{\mu_1 - \mu_0} = \frac{(1.28 + 1.96)^2 (0.109^2 + 0.14^2)}{(1.22 - 1.11)}$$

Using serum calcium mean levels of 1.22 (control) and 1.11 (pregnant women) in a study by Ikekpeazu *et al*., 2010 in Enugu;

u = 1.28 (power = 90%)

v = 1.96 (α level = 5%)

95% Confidence Interval (CI)

Where:

O_1 = the standard deviation for group 1

O_2 = the standard deviation for group 2

μ_1 = the mean for group 1

μ_2 = the mean for group 2

Using OpenEpi, version 3 open source calculator, the calculated sample size for the study was 112 subjects.

3.7 Ethical Approval

Approval was obtained from the Scientific and Ethical Committee on Human Research, ABUTH, Shika, Zaria (Reference number: ABUTHZ/HREC/K30/2014) and consent to participate in the study was sought using standard protocol. These tests were conducted for free.

3.8 Anthropometric Measurements

At the clinics, the women were briefed about the nature of the study and its importance and the subjects who volunteered were served a proforma to fill. Notes from their antenatal folder (for the pregnant subjects) were made after which those who fit into the inclusion criteria were recruited for the study. Using a stadiometer, their height and weight were taken and recorded, then they were taken to the laboratory and blood samples obtained from them.

3.9 Sample Collection and Processing

The subjects were asked to sit down and relax; the antecubital fossa on the arm was then cleaned using methylated spirit and cotton wool and allowed to dry. Slight pressure was applied to the upper arm, temporarily occluding the antecubital vein to identify it. As soon as the vein was visible, using a syringe with a wide bore needle, 10 ml of blood was withdrawn slowly (to avoid bubbles) and transferred to clean plain

serum bottles after removal of the needle and allowed to stand at room temperature (27^{o}C) for two hours so that it could clot.

After two hours the blood samples were centrifuged at 3000 *g* for 5 minutes. The serum was separated to minimise the effect of red cell haemolysis. Blood samples with any degree of haemolysis were considered unsuitable for the study and discarded. The separated serum was stored at -20°C in a deep freezer at the department of Chemical pathology until needed for assay. When needed, samples were thawed at room temperature before analysis, which was completed within two days.

3.9.1 Estimation of serum calcium

(a) *Principle*

The serum calcium assay was carried out at the department of Chemical Pathology, ABUTH, Shika, Zaria. Ionized calcium was estimated using audicom auto-electrolytes analyzer (Model: AC9900). It is an ion selection electrode method in which different concentration of two solutions containing the same ion are separated by a membrane to which the ions are permeable. A potential difference builds up between the two solutions. A metal is in contact with its ion in solution which tends to give up electrons thereby creating a positive charge around its surface in solution. The electrons in solution tend to be deposited on the surface of the metal creating a negative charge on the metal surface. The resulting potential is the difference between the positive and negative charge thus created. Two calibrating solutions A and B were used to establish a calibration curve within the instrument and the ionic concentration in sample was calculated from the calibration curve established (Hawk *et al*., 1951).

(b) *Procedure*

Three hundred microliter (300 µl) of serum was pippeted into glass tubes already provided by the manufacturers of the machine. After pressing analyser button, 160 µl of the serum was automatically pipetted by the autoanalyser into the electrode chamber. After 90 seconds the result was printed out automatically by the instrument inbuilt thermal printer.

3.9.2 Estimation of serum inorganic phosphate

(a) *Principle*:

Inorganic phosphate reacts in acid medium with ammonium molybdate to form a phosphomolybdate complex with yellow colour. The intensity of the colour formed is proportional to the inorganic phosphorus concentration in the sample (Gomori, 1942).

(b) *Procedure*

Three clean centrifuge tubes were labelled test, standard and blank. Ammonium molybdate and sulphuric acid (1000 µl) were added to all three tubes. Serum (10 µl) and standard (10 µl) were added to tubes labeled test and standard, respectively. The content was thoroughly mixed and incubated for 5 min at room temperature (27^{o}C). Absorbance of the samples and standard against the blank were then read at 340 nm. The concentration of serum phosphorus was calculated as follows:

Serum phosphate = A test/A std x Cstd

Where A= absorbance, C= concentration, Std= standard

3.9.3 Estimation of serum 1, 25 (OH) hydroxyvitamin D

25 (OH) Hydroxyvitamin D was assayed using a commercial enzyme immunoassay kit (Wkea, China) according to the manufacture's protocol. The normal range is 30.0 to 74.0 nanograms per milliliter (ng/mL). Assay range of between 50μg/L -1000μg/L

a) *Principle*

The kit assays human vitamin D levesl in the sample using purified human vitamin D antibody to coat microtiter plate wells. It makes a solid-phase antibody, then vitamin D is added to wells. The combined vitamin D antibody which is enzyme labeled, becomes an antibody - antigen - enzyme-antibody complex. After washing completely, the substrate (in this case, serum) is added and it turns blue. As it is HRP enzyme-catalyzed, the reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of vitamin D in the samples is then determined by comparing the optical density of the samples to the standard curve.

b) *Procedure*

The sample was diluted and added to standard: 10 standard wells were set on the microtiter coated plate, 100 μl standard was added to the first and second wells, then 50 μl standard dilution was added to the first and the second wells and mixed. 100 μl was then taken out from the first and second wells and added to the third and forth wells separately. Standard dilution 50 μl was then added to the third and the forth well, then mixed. 50 μl was then removed from the third and the forth well and discarded. 50 μl was added to the fifth and the sixth well, then standard dilution 50 μl was added to the fifth and the sixth well, mixed up to the ninth and tenth wells. 50 μl of sample was

finally added to each well after diluting, (density: 900 μg/L, 600 μg/L, 300 μg/L, 150 μg/L, 75 μg). Setting the blank wells separately, 40μl sample dilution was added to sample well, then 10μl sample was added and gently mixed.

After closing the plate with closure plate membrane, samples were incubated for 30 minutes at 37^{o}C. The incubation mixture is removed by aspirating contents of the plate into a sink or proper waste container. Using a squirt bottle, each well was filled completely with wash solution, then the contents of the plate were aspirated into a sink. The procedure was repeated four more times for a total of five washes. After final wash, the plate was inverted and blot dried by hitting plate onto absorbent paper until there was no more moisture. 50 μl of enzyme conjugate reagent was added to each well, except blank well. Incubation is repeated for 30 minutes at 37°C, removed and washed again five times. To get the colour again, 50 μl of Substrate A and Substrate B were added to each well, covered and incubated for 15 minutes at 37°C. To stop the reaction, 50 μl of stop solution was added to each well and mixed thoroughly. The optical density of each well was determined within 15 minutes by a microplate reader.

Calculation: With the standard density values on the horizontal, the optical density (OD) values on the vertical, a standard curve was drawn on graph paper, the corresponding density according to the sample OD value of the sample curve is determined.

Standard Density x Dilution Factor = Actual Density

3.9.4 Estimation of serum parathyroid hormone

The serum PTH level was measured using the Human Parathyroid hormone (PTH) ELISA Kit (Wkea Medical Supplies Corporation, China), with an assay range of 16 pg/ml -450 pg/ml.

(a) *Principle*

The Intact PTH Immunoassay is a two-site ELISA (Enzyme-Linked ImmunoSorbent Assay). In this assay, standards, controls, or samples are simultaneously incubated with the enzyme labelled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell was washed to remove unbound components and the enzyme bound to the solid phase was incubated with the substrate. An acidic stopping solution was then added to stop the reaction and the colour was converted to yellow. The intensity of the yellow colour was directly proportional to the concentration of intact PTH in the sample. A dose response curve of absorbance unit versus concentration was generated using results obtained from the calibrators. Concentrations of intact PTH present in the controls and samples were determined directly from this curve.

(b) *Procedure*

The diluted sample was added to the standard after setting 10 standard wells on the coated microtiter plate, 100µl of standard was then added to the first and the second well, then 50 µl standard dilution was added to the first and the second well, and mixed. 100 µl was taken out of the first and the second wells and added to the third and the forth well separately, then 50 µl standard dilution was added to the third and the forth wells and mixed. 50 µl was taken out from the third and the forth well separately

and discarded. Another 50 µl was taken from the third and the forth well separately again and added to the fifth and the sixth well separately. This procedure was continued up to the ninth and tenth wells. After diluting, 50 µl was kept in each well (density: 360 pg/ml, 240 pg/ml , 120 pg/ml, 60 pg/ml, 30 pg/ml). The sample is not added to the blank wells and enzyme conjugate. 40 µl of the sample dilution is added to the sample well, then 10 µl of sample is added (sample final dilution is 5-fold), ensuring that the wall and bottom of the well is not touched during addition of samples, then mixed gently.

After covering the plate with closure plate membrane, it was incubated for 30 minutes at 37^{o}C. Afterwards, the incubated mixture was removed. Using a squirt bottle, each well was filled completely with wash solution, then the contents of the plate are discarded into a sink. The procedure was repeated four more times for a total of five washes. After the final wash, the plate was inverted, and blot dried by hitting plate onto absorbent paper until there was no more moisture. 50 µl of enzyme conjugate reagent was added to each well, except the blank well. 50 µl of enzyme conjugate reagent was added to each well, except blank well. Incubation is repeated for 30 minutes at 37°C, removed and washed again five times.

To get the colour again, 50 µl of Substrate A and Substrate B were added to each well, covered and incubated for 15 minutes at 37^{o}C. To stop the reaction, 50 µl of stop solution was added to each well and mixed thoroughly. The optical density of each well was determined within 15 minutes by a microplate reader.

Calculation is done as with vitamin D.

3.9.5 Estimation of serum calcitonin

(a) *Principle*

The kit assays human calcitonin level in the sample, using purified human calcitonin to coat microtiter plate wells, making a solid-phase antibody, followed by adding calcitonin to wells; combined calcitonin antibody which with enzyme labelled, becomes antibody - antigen - enzyme-antibody complex. After washing completely, the substrate is added, then the substrate turns blue. The HRP enzyme-catalysed reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of calcitonin in the samples is then determined by comparing the optical density of the samples to the standard curve.

(b) *Procedure*

The procedure is similar to that of vitamin D and Parathyroid hormone assay but using density of 600 ng/L, 400 ng/L, 200 ng/L, 100 ng/L and 50 ng/L. The calculations were also done as described for vitamin D and parathyroid hormone in sections 3.9.3 and 3.9.4.

3.9.6 Determination of serum antioxidants

3.9.6.1 Oxidative stress profiling

i. Assessment of lipid peroxidation

Cellular integrity was determined by lipid peroxidation as thiobarbituric acid reactive substances, (TBARS), according to Ohkawa *et al*., (1979), as modified by Atawodi *et al*. (2011) using trichloroacetic acid (TCA) and thiobarbituric acid (TBA).

(a) *Principle*

Lipid peroxidation generates peroxide intermediates, which, upon cleavage, releases malondialdehyde (MDA). The MDA is a product that reacts with thiobarbituric acid, to produce a colored complex which absorbs light at a wavelength of 535 nm, which can then be read.

(b) *Procedure*

Test tubes were labeled according to the sample numbers, 1 ml of 15 % TCA added, followed by 1 ml of 0.67 % TBA. The serum samples (50 µL) were then added into corresponding test tube, and the mixture was placed inside a water-bath, kept at 80^{o} C, and incubated for thirty minutes, and allowed to cool. It was then centrifuged at 3,000 rpm for ten minutes, by using the centrifuge Labofuge 300, Heraeus. The clear supernatant was collected. Absorbance of the supernatant was then measured at 535 nm using a spectrophotometer (Jenway 6305).

Calculation: TBARS concentrations were expressed in nmol/mg protein protein, which was calculated as follows:

Concentration mmol/ mg protein = Absorbance of sample/ 1.56 x 10-5 x protein concentration (mg)

The MDA that was formed was calculated by using the molar extinction coefficient of 1.56 x 105 cm-1M-1.

Concentration of MDA = absorbance of sample/1.56 x 105 cm-1 M-1

ii. Determination of serum catalase activity.

(a) *Principle*

Catalase activity was measured by using the method of Aebi (1974).

(b) *Procedure*

Potassium phosphate buffer (50mM), 2.8 ml at pH of 7.0, was added into labeled test tubes. 10 µL of each serum sample were added into the test tubes containing the buffer. The reaction was initiated by adding 0.1 ml of freshly prepared 30 mM hydrogen peroxide, H_2O_2, was then added into each test tube, in batches of twenty, and incubated for 5 minutes. The decomposition rate of the H_2O_2 was measured at 240 nm, using a spectrophotometer (Jenway 6305).

Calculation: A molar extinction coefficient (E) of 0.041 mM-1 cm-1 was used to calculate the activity of catalase.

Catalase concentration = absorbance of sample/molar extinction coefficient x protein concentration (mg/ ml).

One unit is the amount of catalase that decomposes 1mmol of H2O2 per minute at pH of 7.0.

iii. Determination of serum glutathione peroxidase activity

Concentration of reduced glutathione was determined according to Ellman (1959), as described by Rajagopalan *et al*., (2004).

(a) *Principle*

This test is based on the reaction of 5, 5'-dithiobis nitro benzoic acid (DNTB) and reduced glutathione (GSH).

(b) *Procedure*

Serum sample (150 µL) was added into correspondingly labeled test- tubes, 1.5 ml of 10 % TCA was added into each tube and centrifuged at 1500 g for 5 minutes. The supernatant (1 ml) was treated with 0.5 ml of Ellman's reagent, and 3 ml of phosphate buffer at pH 8.0. Absorbance was read at 412 nm. The quantity of GSH was extrapolated from a graph of the GSH standard curve.

iv Determination of superoxide dismutase activity.

Superoxide dismutase (SOD) activity was determined by the method described by Fridovich (1989).

(a) *Principle*

The principle is based on the ability of SOD to inhibit auto-oxidation of adrenaline at pH 10.2.

(b) *Procedure*

Serum sample 0.1 ml was diluted with 0.9 mL of distilled water, to make 1: 10 dilution of microsome. 0.2 mL of the diluted mixture was added to 2.5 mL of 0.05M carbonate buffer, and then 0.3 mL of 0.3 nM adrenaline was added to start the reaction. 2.5 mL of 0.05 M carbonate buffer was mixed with 0.3 mL of 0.3 mM adrenaline and 0.2 mL of distilled water in a separate test tube. This was the reference mixture. Absorbance was measured over 30s up to 150s at 480 nm.

Calculation: Increase in absorbance per minute = (A5- A1) / 2.5

$$\% \text{ inhibition} = \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 100$$

One unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in one minute.

3.10 Quality Control

The samples were analysed along with quality control materials and factors which could affect the values obtained from the study were avoided such as:

i. Outliers: though these were few, they were excluded from the final computation of data.

ii. Sample size: adequate samples were collected to make provision for artrition and ensure a good statistical outcome.

iii. Method of sample collection: The use of tourniquet was avoided in blood sample collection to ensure correct ionised calcium values were obtained.

3.11 Data Analysis

Data were entered into Microsoft excel and were presented as mean ± SD. Analysis of data was done using One Way ANOVA to find the significance between the groups followed by Holm-Sidak post hoc - test. Pearson's correlation analysis was done to determine the inter-relationship between the variables. Values of $p \leq 0.05$ were considered significant. Statistical package used was Minitab statistical software version 16.

CHAPTER FOUR

4.0 RESULTS

4.1 Anthropometric Data

The anthropometric indices of the subjects are shown in Table 4.1. The study showed that the highest mean age was in the control group and, the lowest was in the 3rd trimester. The mean highest mean weight was in the pregnant women in the 3rd trimester while the lowest was in the lactating group. The highest mean height was among the 2nd trimester pregnant women and the lowest was in the women in their 1st trimester of pregnancy. The highest mean BMI was in the the control group while the lowest was in pregnant women in the lactating group of women.

4.2 Serum Levels of Calcium, Vitamin D, Parathyroid Hormone, and Calcitonin

Table 4.2 shows the profile of mean calcium, phosphate (PO_4), vitamin D, parathyroid hormone (PTH) and calcitonin in the control, pregnant and lactating women. The highest mean value for serum calcium was found in pregnant women in the 1st trimester while the lowest was found in the lactating group and this was significant. Serum phosphate concentration was highest in pregnant women in the control group and lowest in the 1st trimester. Serum vitamin D and parathyroid hormone concentrations were highest in pregnant women in the 2nd trimester and lowest in the control group and 1st trimester respectively.

Table 4.1: Table of anthropometric indices of control, pregnant and lactating women in Zaria, Nigeria

Parameters	**Age (yrs)**	**Weight (kg)**	**Height (cm)**	**BMI (Kg/m^2)**	**Gest. Age(mths)**
Control (n = 15) non-pregnant	29.79±5.56	67.40±9.49	159.27±9.13	26.56±3.12	-
1^{st} trimester (n = 40)	27.00±6.41	63.39±16.91	157.10±8.29	25.32±6.98	2.71±0.49
2^{nd} trimester (n = 41)	28.17±6.11	66.42±14.53	161.78±7.12	24.58±7.06	5.42±0.81
3^{rd} trimester (n = 53)	26.62±5.58	68.39±13.04	159.82±6.52	24.00±8.35	7.74±0.79
Lactating gp (n = 30)	28.37±5.13	63.13±12.82	160.90±7.38	24.32±4.18	-

Key: n – number of women per group, Gest. Age – Mean gestational age, BMI – Body Mass Index

4.3 Prevalence of Calcium, Phosphate, Vitamin D, Parathyroid Hormone, and Calcitonin Deficiency

Figure 4.1 shows 1.76% of pregnant women with calcium below the reference range and 5.31% above the reference range in this study. 1.76% of them had phosphate levels below the reference range and none of them was hyperphosphataemic. Vitamin D concentration was below reference in 6.19% and above the reference range in 21.24% of the pregnant women. Parathyroid hormone level was below reference in less than 1% (one per cent) of the study population and 7.97% above the reference range. Calcitonin was not below the reference range in any of the groups, but there was a 1.76% prevalence above the reference range.

4.4 Relationship between Gestational Age and Serum Calcium Concentration

Figure 4.2 shows a scatterplot of gestational age against calcium. It was observed that there was a negative correlation ($r = -0.255$) between calcium and gestational age i.e. as pregnancy progressed, there was decreased serum concentration of calcium and this decrease was statististically significant ($P < 0.05$).

4.5 Relationship between Gestational Age and Serum Phosphate Concentration

Figure 4.3 shows a scatterplot of the relationship between gestational age and serum phosphate concentration. There was a positive correlation between phosphate and gestational age i.e. as pregnancy progressed, there was increased serum concentration of phosphate, but this increase was not statistically significant ($P > 0.05$).

Table 4.2: Mean (±SD) Serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin in control, pregnant and lactating women in Zaria, Northern Nigeria.

Parameters	**Control (n = 15)**	**1st Trim (n = 40)**	**2nd Trim (n =41)**	**3rd Trim (n =53)**	**Lactating Gp (n =30)**	**P value**
Calcium (mmol/L)	2.30±0.10	2.32±0.11	2.31±0.15	2.24±0.14**	2.21±0.17**	P = 0.001
PO_4 (mmol/L)	1.13±0.15	1.01±0.13	1.08±0.14	1.08±0.15	1.09±0.14	P = 0.065
Vitamin D (µg/L)	39.29±4.51	41.21±5.68	46.57±20.90	42.44±8.50	43.33±20.08	P = 0.426
PTH (pg/ml)	40.79±8.43	40.66±6.64	45.60±17.62	41.84±10.40	44.81±12.63	P = 0.407
Calcitonin (ng/L)	3.47±0.91	3.05±0.93	3.95±4.10	3.20±0.92	3.10±0.77	P = 0.452

Key: ** Represents P < 0.01 when compared to the control, Trim - Trimester, Gp - Group, PTH - parathyroid hormone

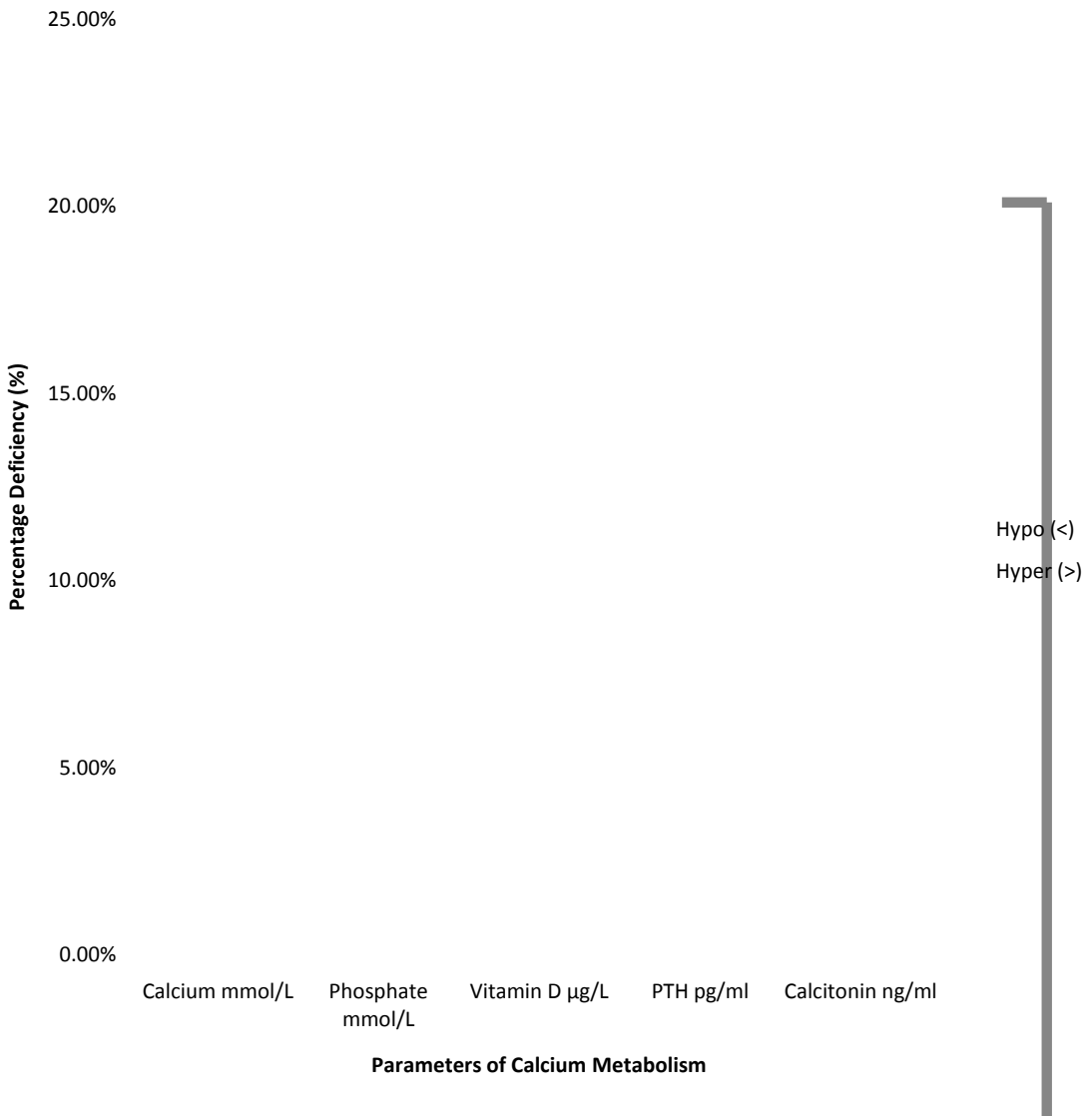


Figure 4.1: Pattern of calcium, phosphate, vitamin D, parathyroid hormone, and Calcitonin metabolism disorders among pregnant and lactating women in Zaria, Nigeria

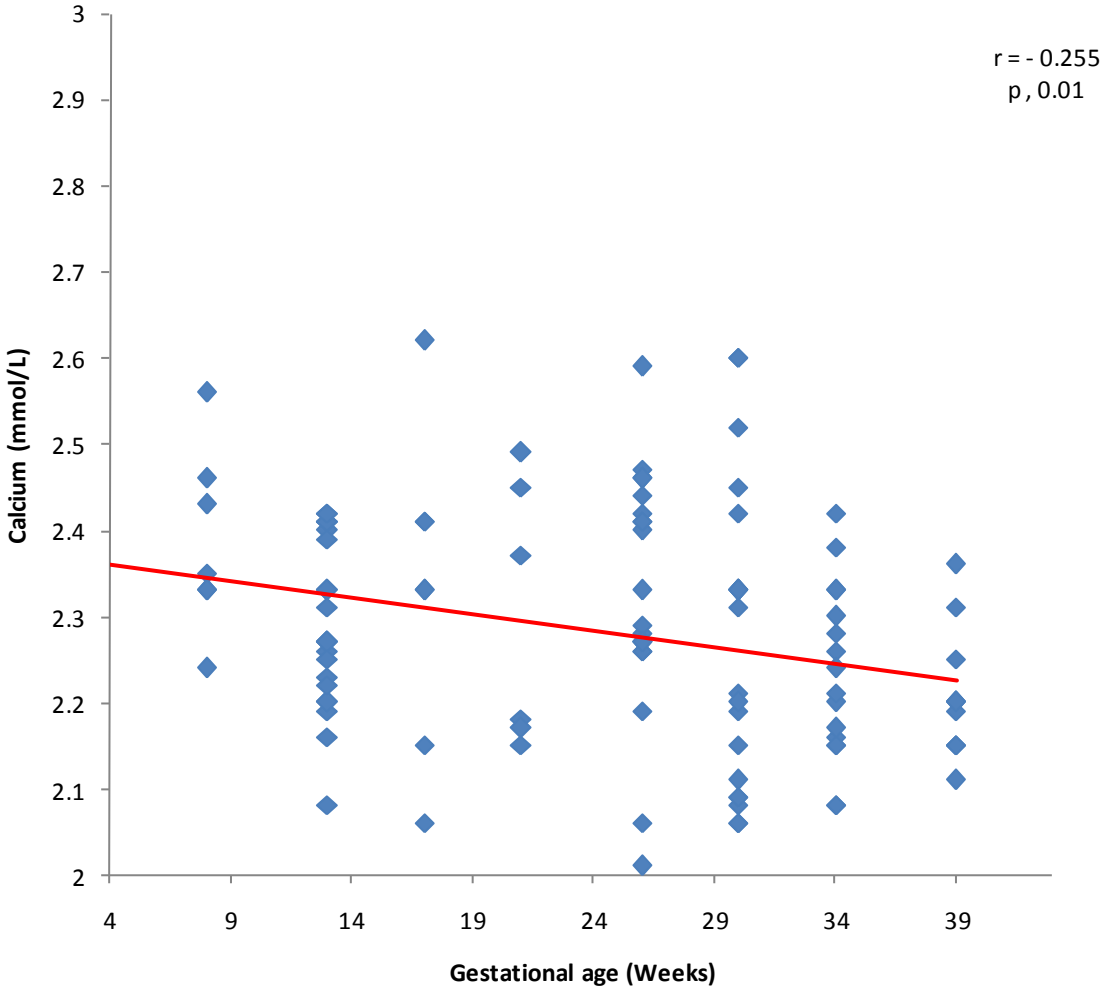


Figure 4.2: Relationship between gestational age and serum calcium concentration (mmol/L)

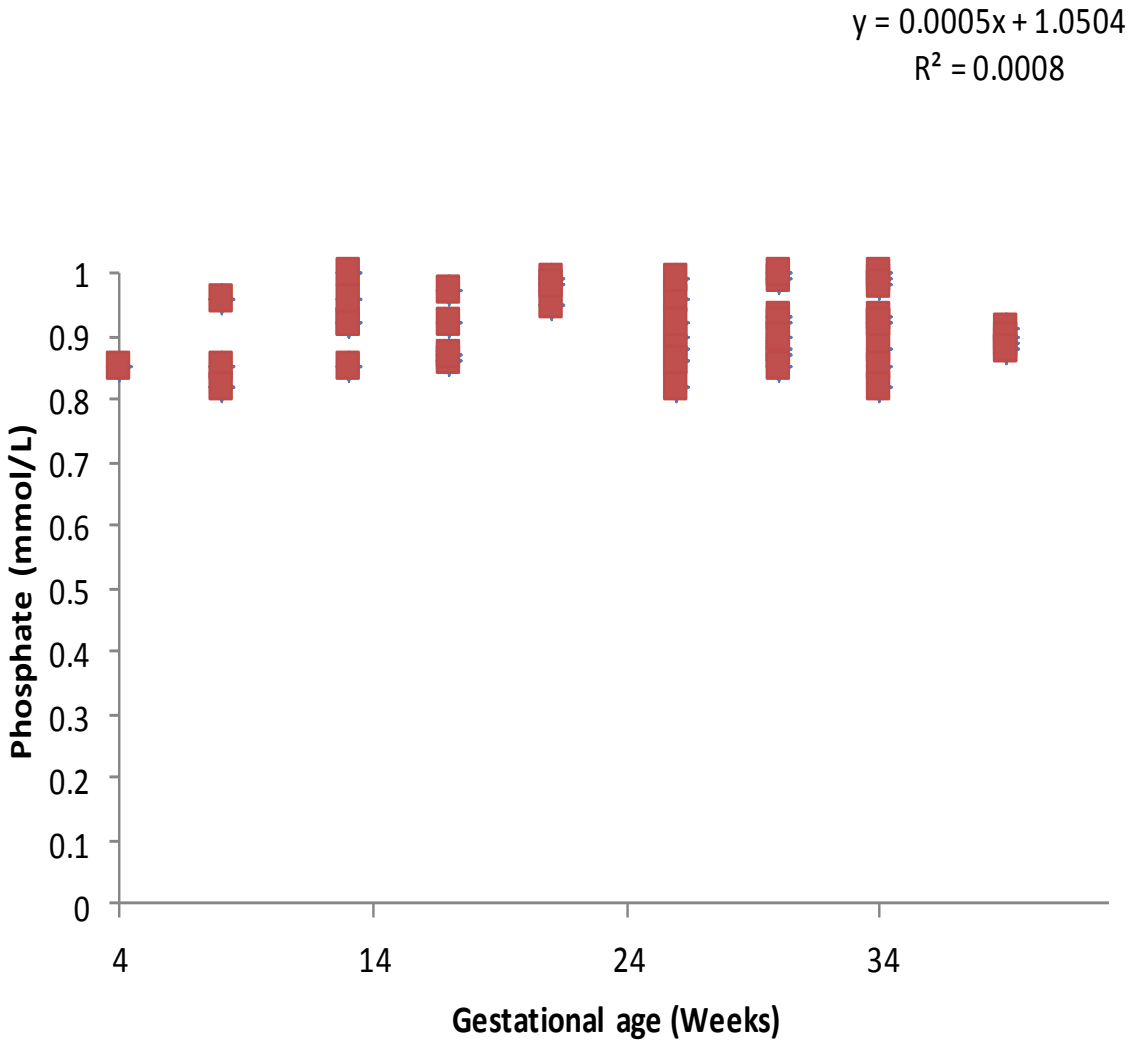


Figure 4.3: Relationship between gestational age and serum phosphate concentration (mmol/L)

4.6 Relationship between Age, Gestational Age, Body Mass Index and Serum Concentration of Calcium, Phosphate, Parathyroid Hormone and Vitamin D in Pregnant and Lactating Women in Zaria, Nigeria

Table 4.3 shows the correlation between age, gestational age, body mass index (BMI), and serum concentrations of calcium, phosphate, parathyroid hormone (PTH), vitamin D and calcitonin. About 20% (twenty percent) of the variables correlated significantly. Positive correlation was observed between age and BMI and parathyroid hormone and vitamin D; while negative correlation was observed between phosphate and calcium, vitamin D and calcium, parathyroid hormone and calcium and calcitonin and phosphate. These correlations were statistically significant ($P < 0.001$).

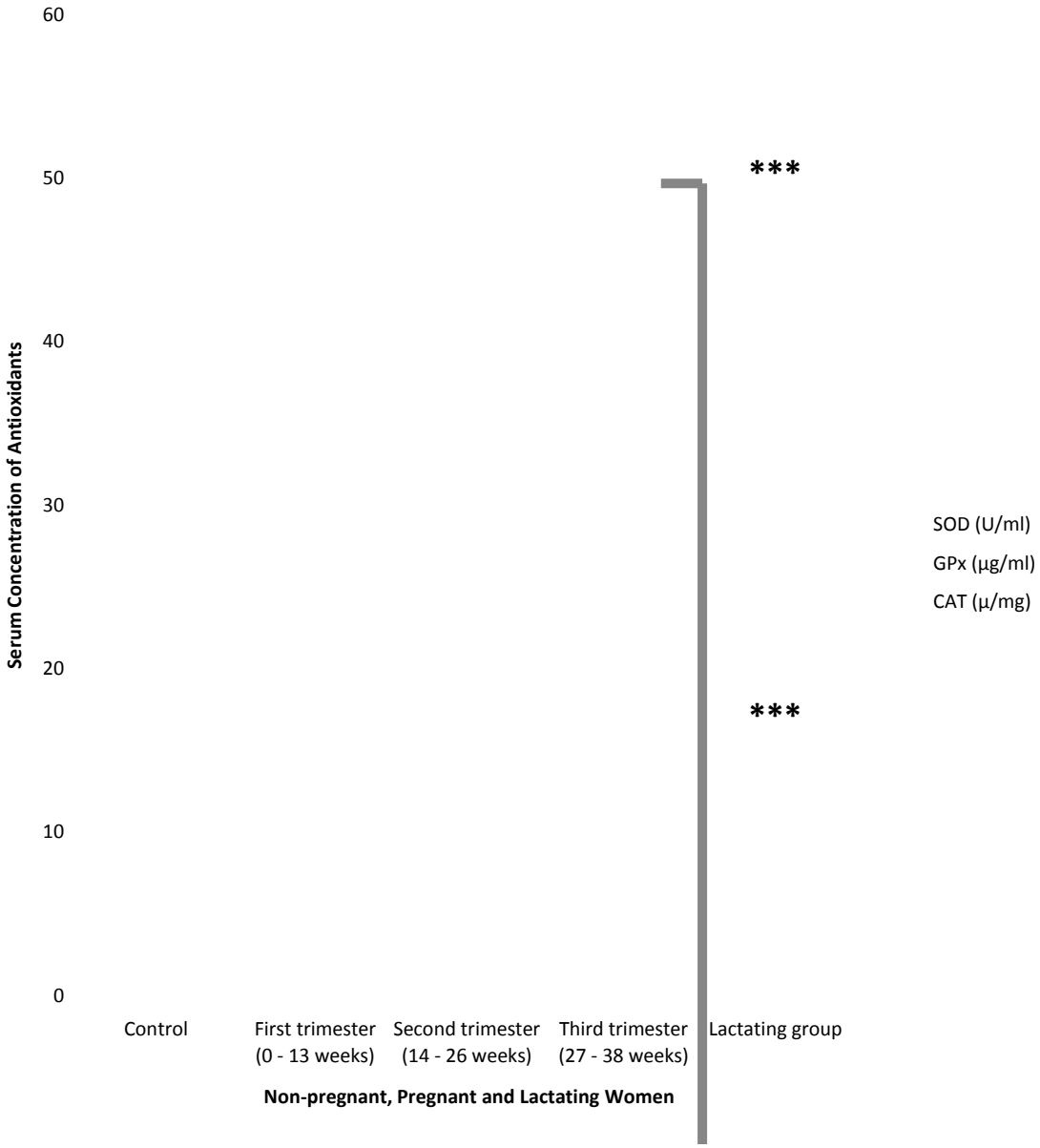
4.7 Mean (±SEM) Serum Concentrations of Malondialdehyde, Superoxide Dismutase, Glutathione Peroxidase and Catalase in pregnant and lactating women in Zaria, Nigeria

Figure 4.4 shows the concentration of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in the serum of subjects. The MDA was highest in the lactating group (349.03±102.25 nmol/mg protein) and this difference was statistically significant ($p < 0.01$). There was a statistically significant ($P < 0.001$) increase observed in the level of SOD between the different groups. This difference was observed in the lactating group, where it was higher, 14.87±1.61U/ml. The GPx was also highest in the lactating group 47.43±26.00ug/ml; and lowest in the control (11.71±1.83 ug/ml), and this difference was statistically significant ($p < 0.05$). The serum level of CAT was highest (17.56±8.81 u/mg) in the 2nd trimester and lowest in the lactating group (3.14±0.78 u/mg), However, the difference was not statistically significant ($p > 0.05$).

Table 4.3: Relationship between age, gestational age, BMI and serum concentration of calcium, phosphate, parathyroid hormone, vitamin D and calcitonin in pregnant and lactating women in Zaria, Nigeria

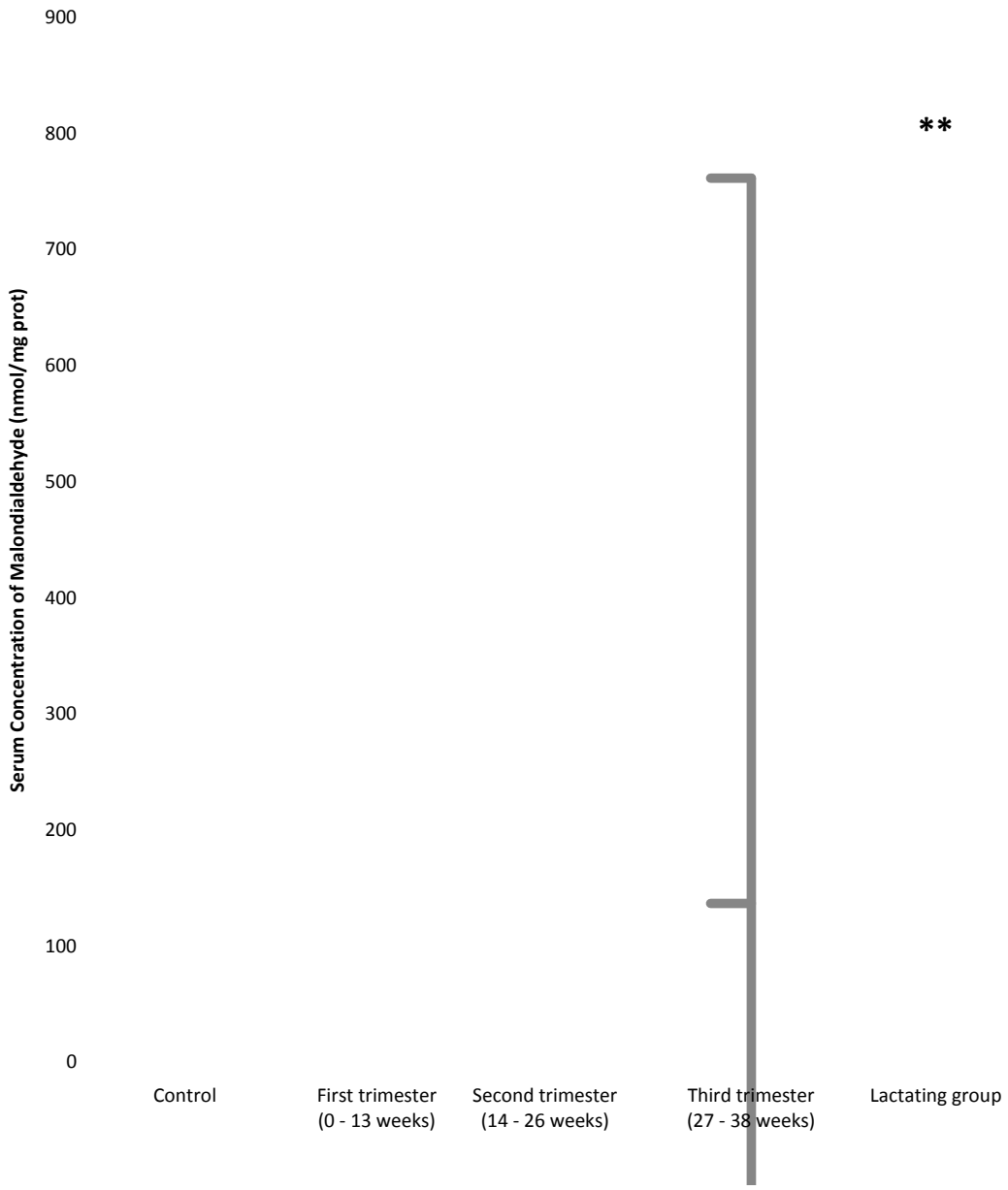
Parameters	R	P value
BMI and Age	0.290	<0.001
Calcium and G. Age	- 0.255	<0.01
Phosphate and Calcium	- 0.270	<0.001
Vitamin D and Calcium	- 0.422	<0.001
PTH and Calcium	- 0.448	<0.001
PTH and Vitamin D	0.756	<0.001
Calcitonin and Calcium	0.279	<0.001
Calcitonin and Phosphate	- 0.239	<0.001

Key: G. Age = Gestational age, wks = weeks, BMI = Body Mass Index, PTH = Parathyroid hormone



Key: *** = $p < 0.001$

Figure 4.4: Pattern of change in serum concentration of superoxide dismutase, glutathione peroxidase and catalase in control, pregnant and lactating women in Zaria, Nigeria



Key: ** = $p < 0.01$

Figure 4.5: Pattern of change in serum concentration of malondialdehyde in control, pregnant and lactating women in Zaria, Nigeria

4.8 Relationship between Gestational Age and Serum Levels of Catalase, Superoxide Dismutase, Glutathione Peroxidase and Malondialdehyde in pregnant and lactating women in Zaria, Nigeria

Table 4.4, it shows the correlation between gestational age (G Age) and serum concentration of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA). Positive correlation was observed between MDA and CAT, GPx and SOD which were statistically significant ($p < 0.05$); while negative correlation was observed between MDA and SOD, MDA and GPx which were also statistically significant ($p < 0.05$).

There was strong positive correlation between vitamin D with superoxide dismutase ($P < 0.05$) and glutathione peroxidase ($P < 0.01$). There was also a negative correlation between serum phosphate concentration and MDA concentration which was statistically significant ($P < 0.05$).

4.9 Regression Equations for Estimating Calcium Levels in Pregnant and Lactating Women in pregnant and lactating women in Zaria, Nigeria

Table 4.6 shows a linear regression estabilishing that depending on the trimester of pregnancy or the period of lactation, serum phosphate and vitamin D levels significantly predicted the serum concentration as follows:

1^{st} Trimester: Calcium = 3.036 – (0.317*Phosphate) – (0.00966 * Vit D) F (7.84); P = 0.002

2^{nd} Trimester: Calcium = 2.784 - (0.253 * Phosphate) – (0.00395 * Vit D) F (10.78); P < 0.001

3^{rd} Trimester: Calcium = 2.766 - (0.228 * Phosphate) – (0.00640 * Vit D) F (7.993); P < 0.001)

Lactating women: Calcium = 2.554 - (0.154 * Phosphate) – (0.00384 * Vit D) F(4.39); P = 0.028

Table 4.4: Relationship between gestational age and serum concentrations of vitamin D, phosphate, catalase, superoxide dismutase, glutathione peroxidase and malondialdehyde in pregnant and lactating women in Zaria, Nigeria

Parameters	R	P value
Gestational Age and Catalase	-0.043	0.66
Gestational Age and Superoxide Dismutase	-0.008	0.94
Gestational Age and Glutathione Peroxidase	-0.011	0.91
Gestational Age and Malondialdehyde	0.022	0.82
Glutathione Peroxidase and Superoxide Dismutase	0.667	0.001
Malondialdehyde and Catalase	0.239	0.01
Malondialdehyde and Superoxide Dismutase	0.326	0.001
Malondialdehyde and Glutathione Peroxidase	-0.580	0.001
Superoxide dismutase and Vitamin D	0.175	< 0.05
Glutathione Peroxidase and Vitamin D	0.227	0.01
Malondialdehyde and Phosphate	-0.189	< 0.05

Table 4.5: Regression Equations for Estimating Calcium Levels in Pregnant and Lactating Women in Zaria, Nigeria

Variables	Predictive Equation	SEE	r	r^2	P
1st Trimester	Calcium = 3.036 – (0.317*Phosphate) – (0.00966 * Vit D)	0.088	0.613	0.376	0.002
2nd Trimester	Calcium = 2.784 - (0.253 * Phosphate) – (0.00395 * Vit D)	0.118	0.634	0.403	<0.001
3rd Trimester	Calcium = 2.766 - (0.228 * Phosphate) – (0.00640 * Vit D)	0.115	0.512	0.262	0.001
Lactating Women	Calcium = 2.554 - (0.154 * Phosphate) – (0.00384 * Vit D)	0.127	0.572	0.328	0.028
Non-Lactating Women	Calcium = 2.455 - (0.0385 * Phosphate) – (0.00266 * Vit D)	0.103	0.148	0.0219	0.895

Key: SEE – Standard error of estimate, r – coefficient of regression, r^2 – coefficient of regression squared,

4.10 Age Distribution and Serum Calcium Concentrations in Controls, Pregnant and Lactating Women

Figure 4.5 shows the age distribution of serum calcium concentration. In the control group, the the highest calcium concentration was observed in the 21 – 25 years, while the lowest was observed in the 26 – 30 years category. During the first trimester, the highest concentration was seen in the 26 – 30 years category and the lowest was in the 16 – 20 years category. The highest value observed in the second trimester was in the 26 – 30 years category, and the lowest was in the 16 – 20 years. In the 3rd trimester, the highest concentration was seen in the 36 – 40 years age groupand the lowest in the 16 – 20 years age group. However, the differences between age groups were not significant ($P > 0.05$).

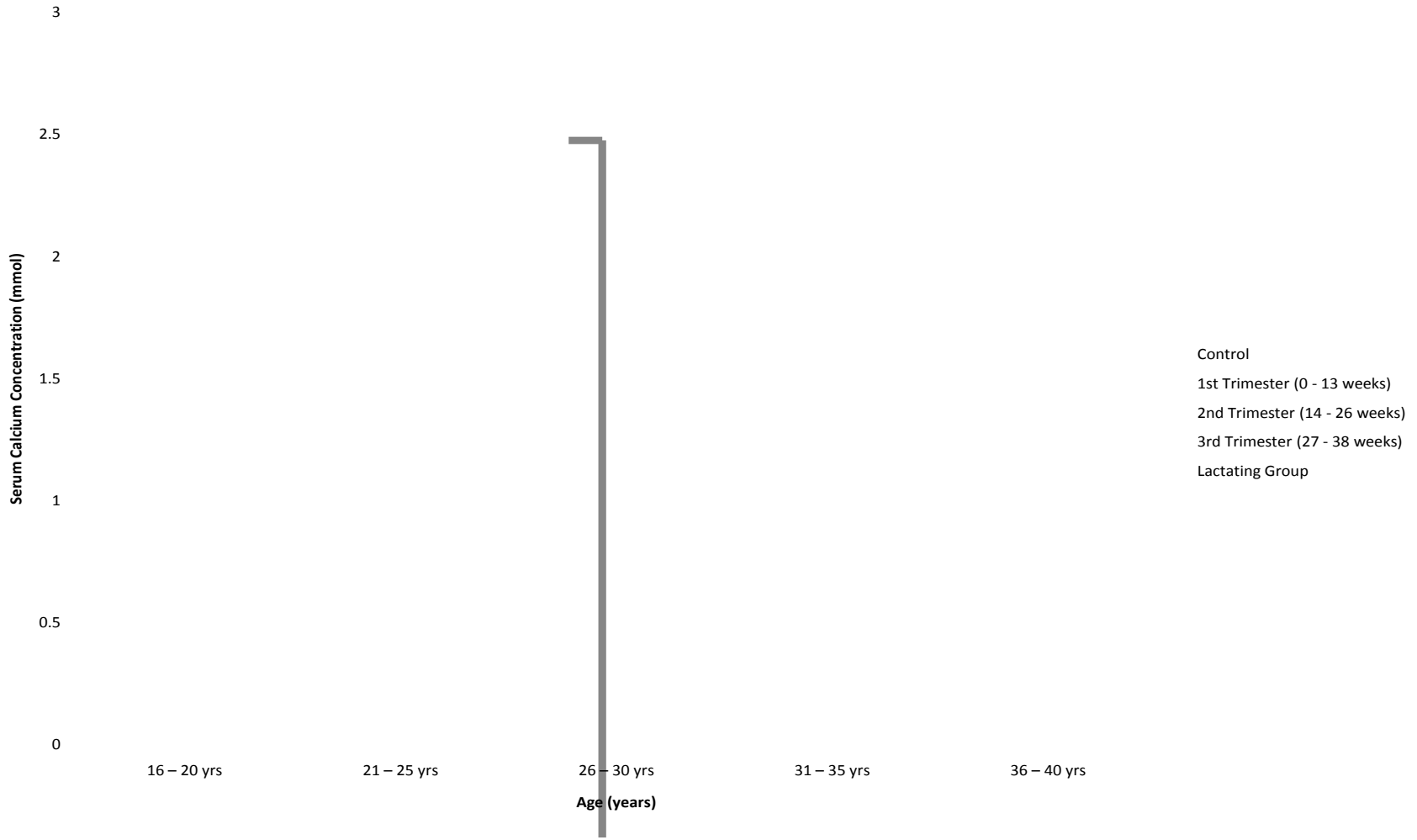


Figure 4.6: Age distribution of mean (±SD) serum concentration of calcium (mmol) in control, pregnant and lactating women in Zaria, Nigeria

4.11 Age Distribution of Serum Phosphate Concentration in Control, Pregnant and Lactating Women

Figure 4.6 shows the age distribution of serum phosphate concentration. In the control group, the highest phosphate concentration was observed in the 21 – 25 age group and lowest in the 31 – 35 age group; while in the 1st trimester, the highest was in the 16 – 20 age group and the lowest was in the 21 – 25 age group. In the 2nd trimester and 3rd trimesters, the highest were in the 36 – 40 age group and in the 16 – 20 age group, respectively. For the lactating group, the highest concentration was found in the 35 – 40 age group and the lowest found in the 16 – 20 age group. However, these differences in serum phosphate concentrations between age groups were not significant ($P > 0.05$).

4.12 Age Distribution of Serum Vitamin D Concentration on Control, Pregnant and Lactating Women

In figure 4.7, age distribution and serum concentration of vitamin D is presented. In the control group, the highest mean value was in the 31 – 35 age group and the lowest value was recorded in the 21 – 25 age group. The differences observed in all the groups were not significant ($P > 0.05$).

4.13 Age Distribution of Serum Parathyroid Hormone Concentration on Control, Pregnant and Lactating Women

Figure 4.8 shows age distribution and serum parathyroid hormone (PTH) concentration. In the control group, the highest PTH concentration was observed in the 26 – 30 age group, while the lowest was observed in the 21 – 25 age group. In the 1st, 2nd and 3rd trimesters, the highest were in the 21 – 25 age group, in the 26 – 30 age group and in the 16 – 20 age group, respectively.. These values were also not significant ($P > 0.05$)

4.14 Age Distribution of Serum Calcitonin Concentration on Control, Pregnant and Lactating Women

Figure 4.9 shows the age distribution of serum calcitonin (CT) concentration. In the control group, the highest calcitonin concentration was observed in the 21 – 25 age group and the lowest was seen in the 26 – 30 age group. In the 1^{st}, 2^{nd} and 3^{rd} trimester groups, the highest concentration was was found in the 35 – 40 age group. While, the lowest serum concentration of calcitonin (CT) in the 1^{st}, 2^{nd} and 3^{rd} trimesters was found in the 25 – 30 age group; in the 26 – 30 age group; and in the 31 – 35 age group, respectively. In the lactating group, the highest and lowest serum calcitonin (CT) concentrations were found in the 16 – 20 age group and in the 26 – 30 age group, respectively. The difference between age groups were not significant ($P > 0.05$).

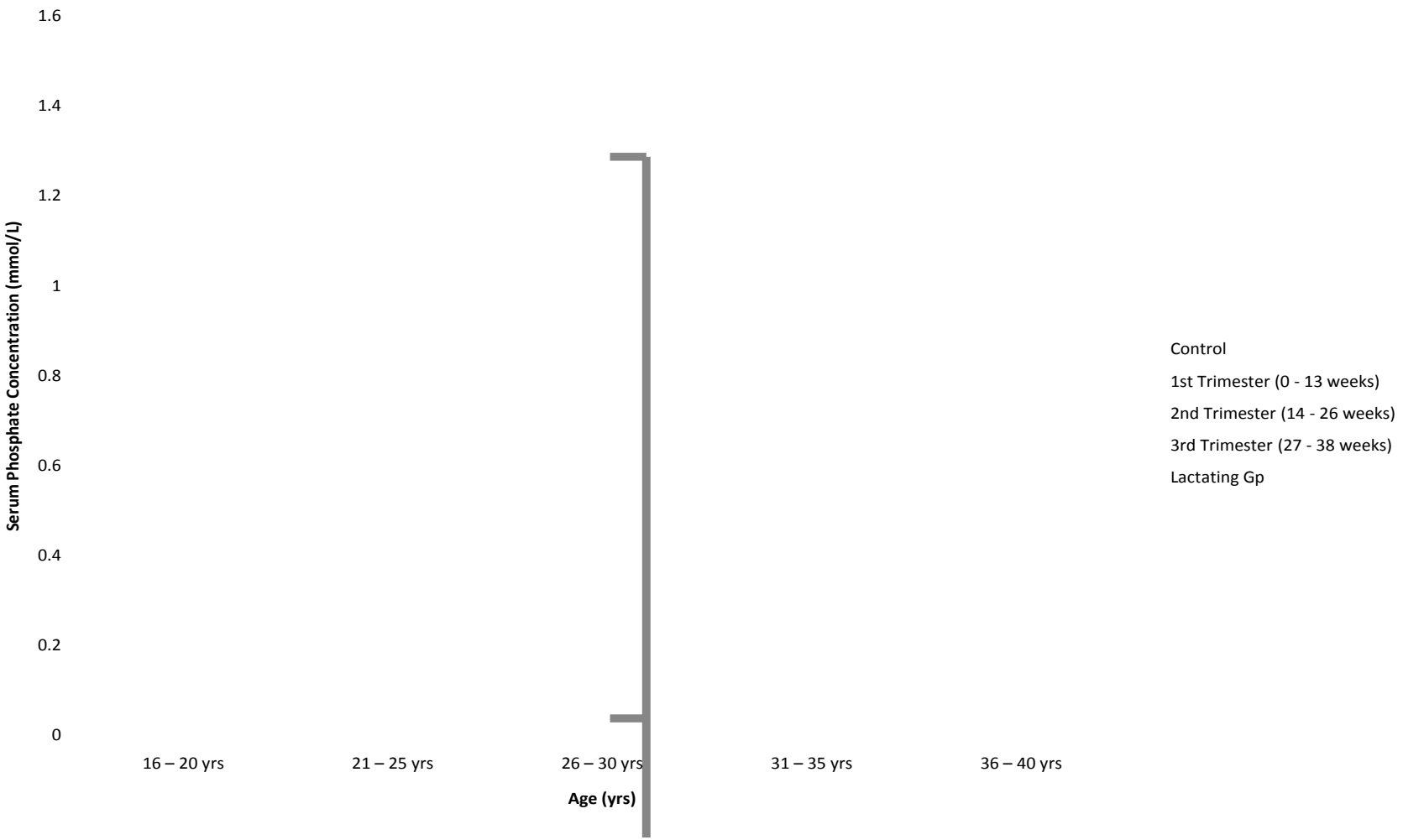


Figure 4.7: Age distribution of serum concentration of phosphate (mmol/L) in control, pregnant and lactating women in Zaria, Nigeria

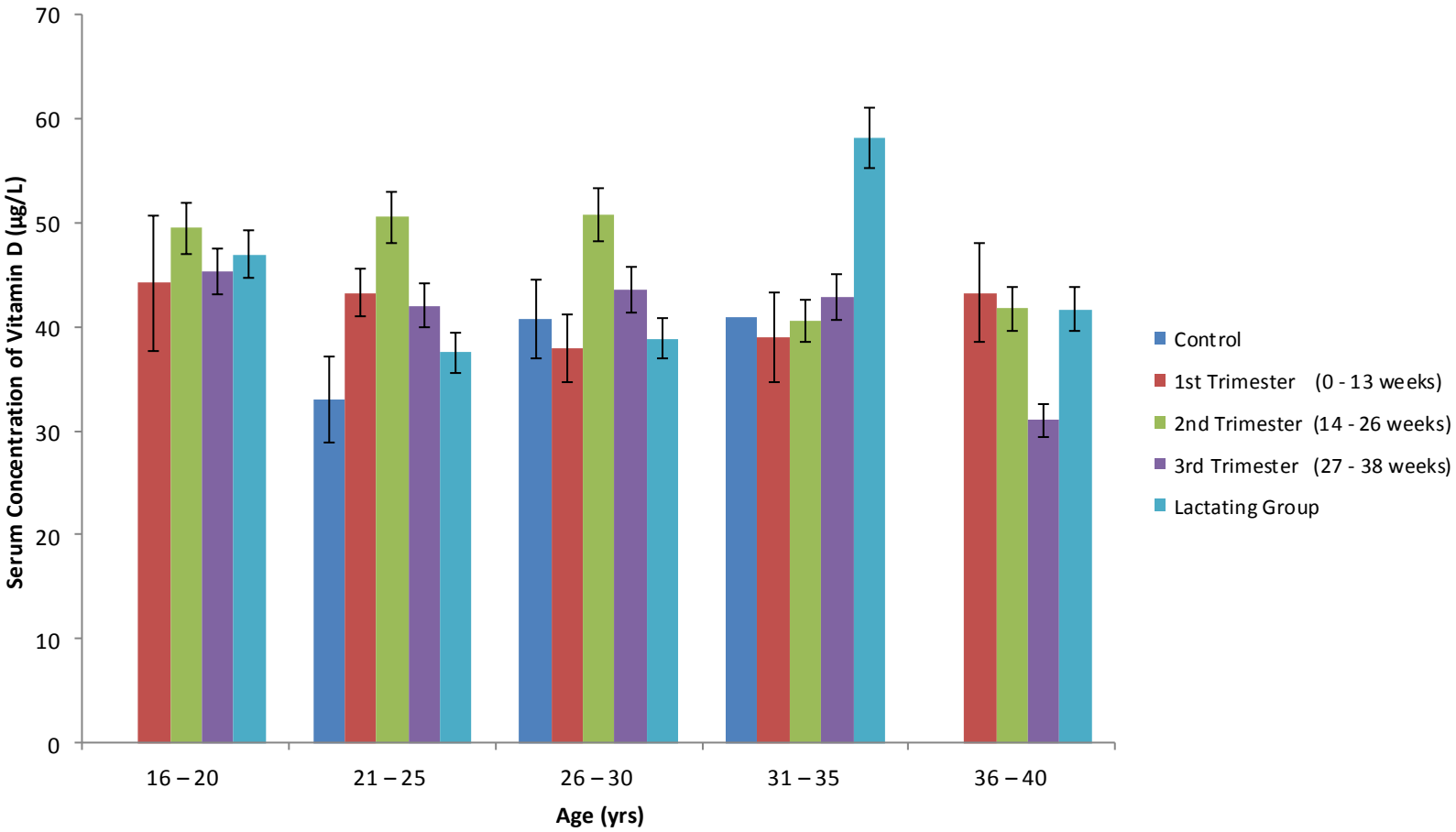


Figure 4.8: Age distribution in serum concentration of vitamin D (µg/l) in control, pregnant and lactating women in Zaria, Nigeria

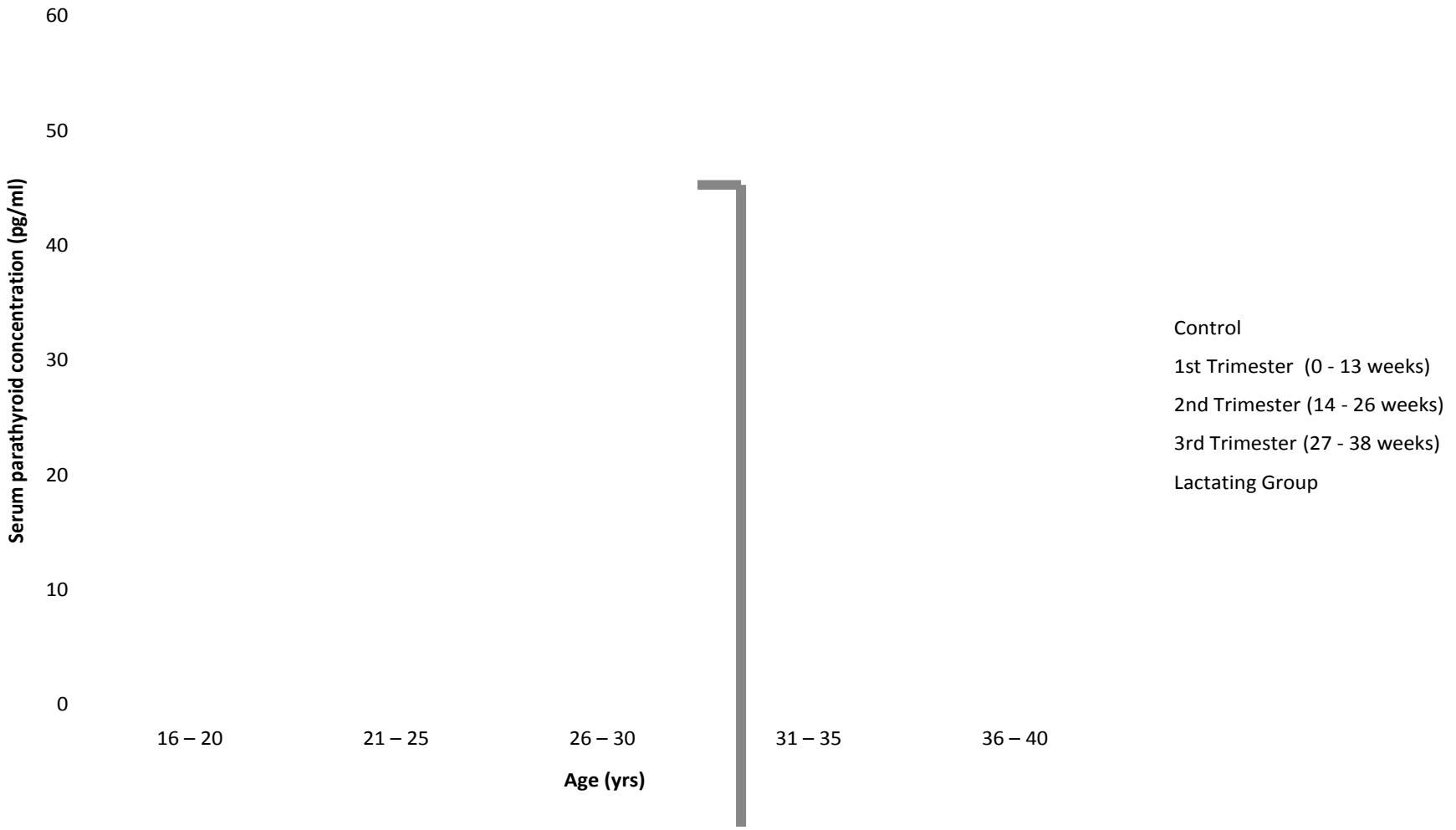


Figure 4.9: Age distribution of serum concentration of parathyroid hormone (pg/ml) in control, pregnant and lactating women in Zaria, Nigeria

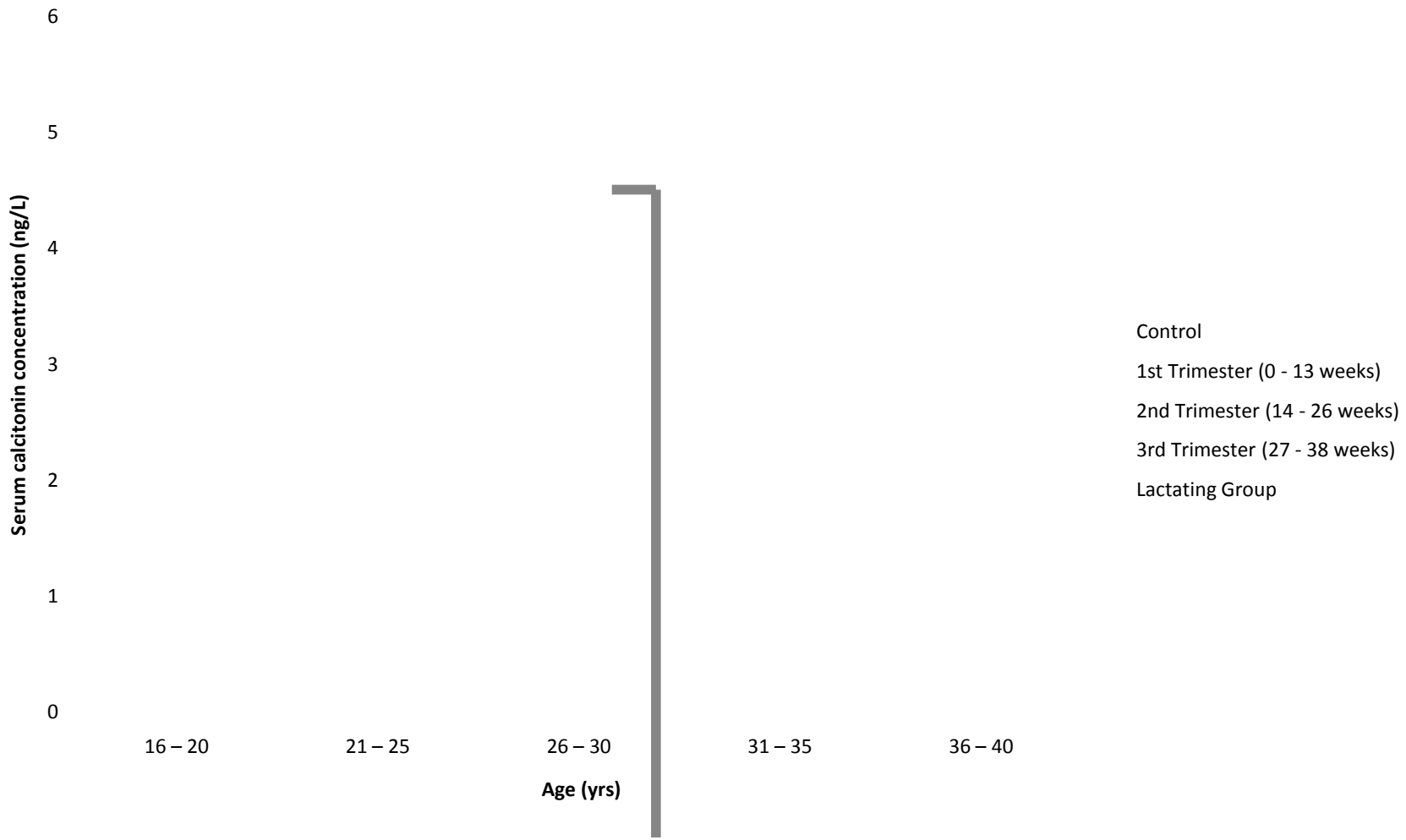


Figure 4.10: Age distribution of serum concentration of calcitonin (ng/L) in control, pregnant and lactating women in Zaria, Nigeria

4.15 Effect of Parity on Serum Concentration of Calcium, Phosphate, Vitamin D, Parathyroid Hormone and Calcitonin

In figures 4.10, 4.11, 4.12. 4.13 and 4.14 show the effect of parity on calcium, phosphate, vitamin D, PTH and calcitonin concentration. Calcium levels were highest in those who had 7 -10 children and lowest in the control group. There was a steady rise in serum calcium level as parity increased, but this was not statistically significant. Phosphate concentration was highest in the control group and lowest in the 4 – 6 parity group. In those who had 4 – 6 children, vitamin D levels were highest and lowest in the 7 – 10 parity group. The serum PTH concentration was highest in the 4 – 6 parity group (45.074±16.35 pg/ml) and lowest in the 7 – 10 parity group. In the control group calcitonin concentratin was highest and lowest in the 0 – 3 parity group. However these differences were not significant ($P > 0.05$).

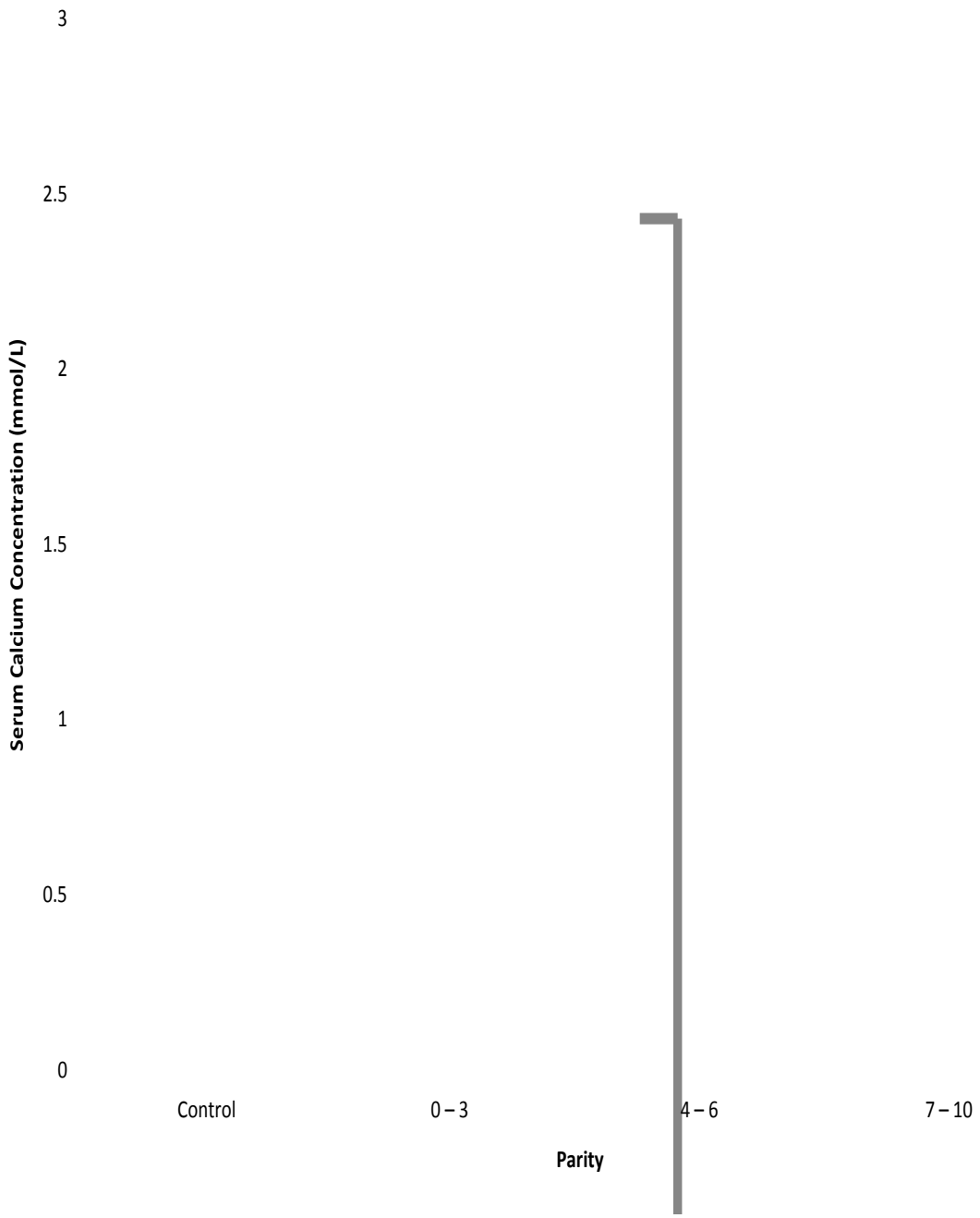


Figure 4.11: Effect of parity on serum concentration of calcium in control, pregnant and lactating women in Zaria, Nigeria.

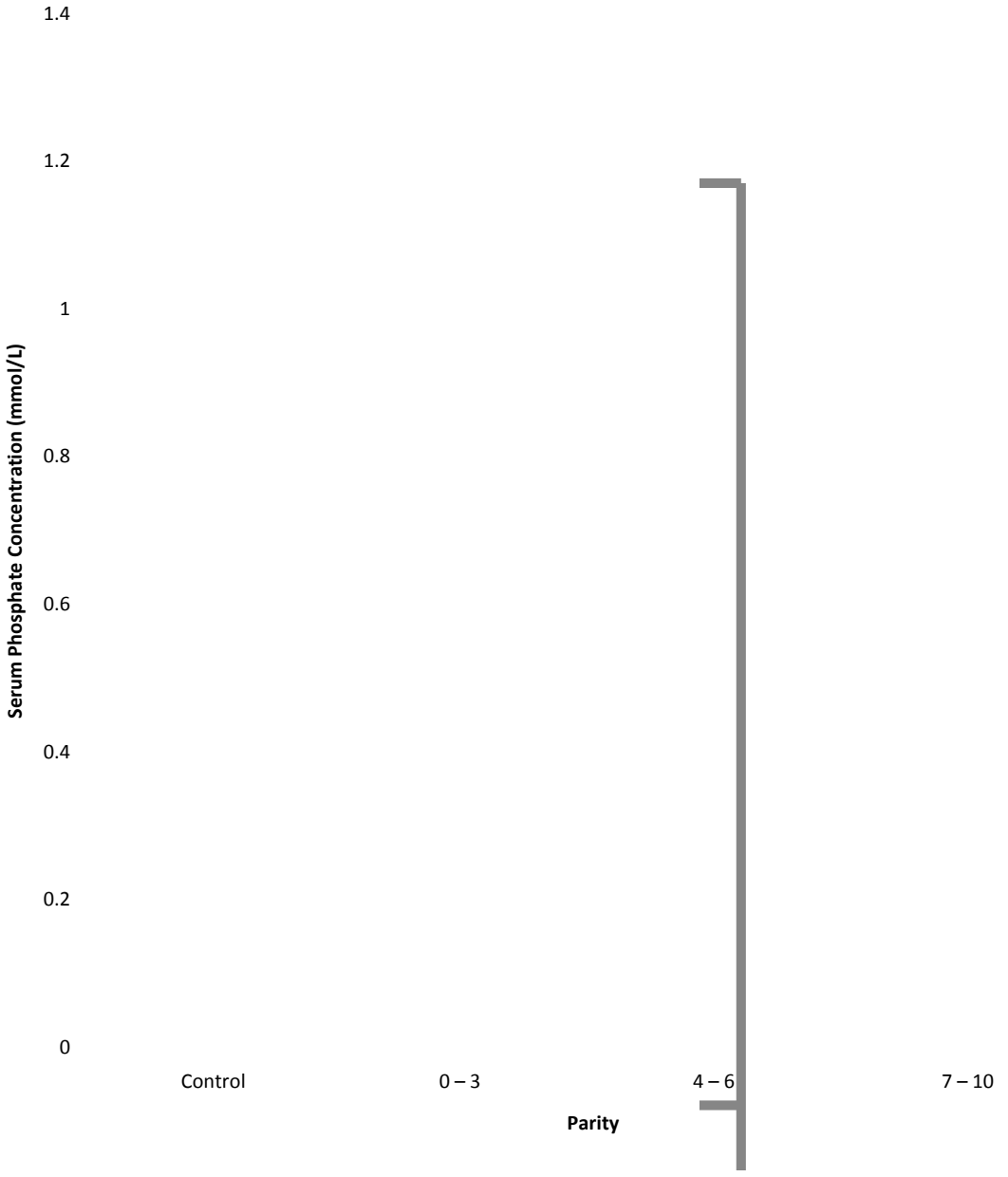


Figure 4.12: Effect of parity on serum concentration of phosphate in control, pregnant and lactating women in Zaria, Nigeria.

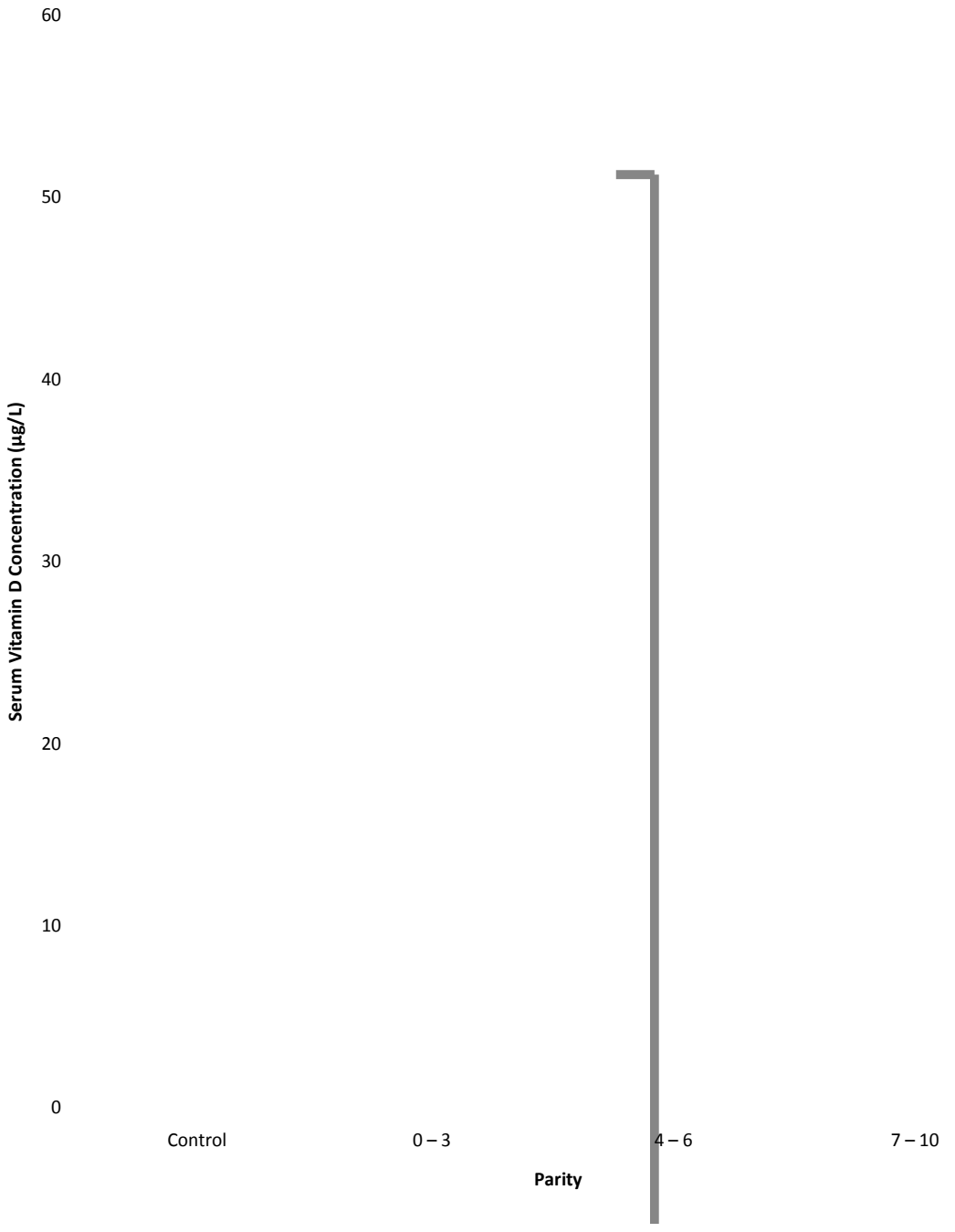


Figure 4.13: Effect of parity on serum concentration of vitamin D in control, pregnant and lactating women in Zaria, Nigeria

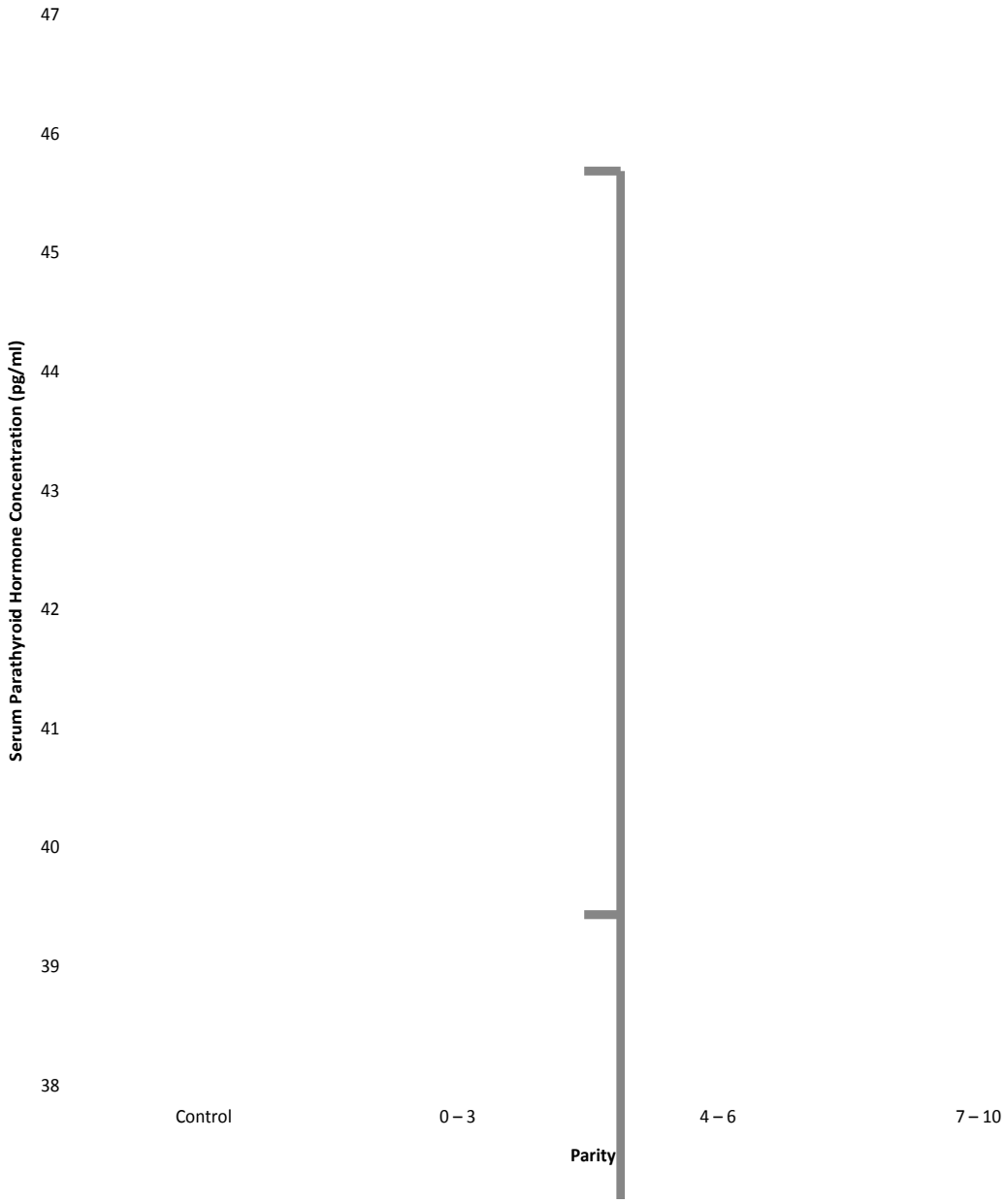


Figure 4.14: Effect of parity on serum concentration of parathyroid hormone in control, pregnant and lactating women in Zaria, Nigeria.

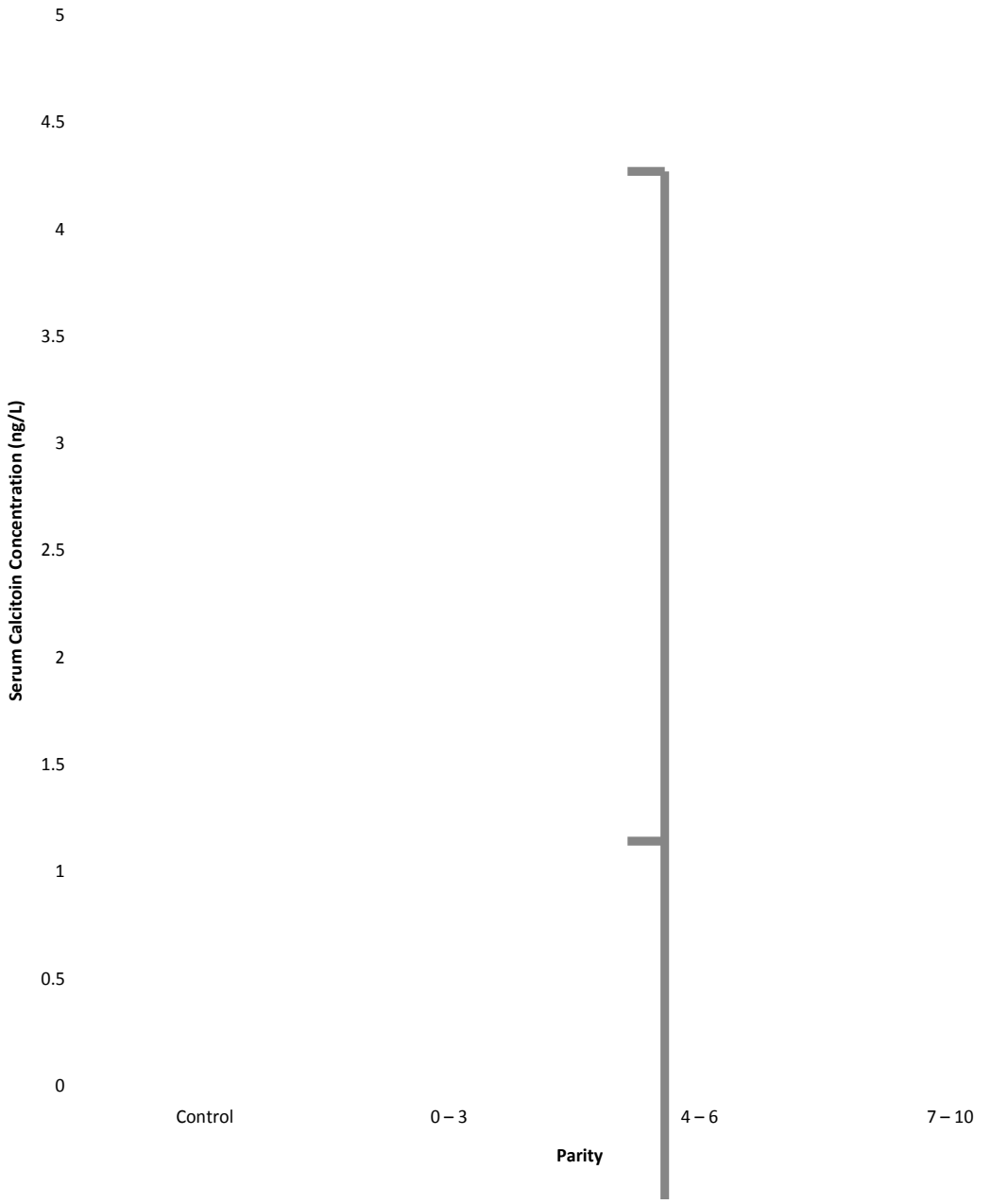


Figure 4.15: Effect of parity on serum concentration of calcitonin in control, pregnant and lactating women in Zaria, Nigeria.

4.16 Influence of Ethnicity on Serum Concentration of Calcium, Phosphate, Vitamin D, Parathyroid Hormone and Calcitonin in Control, Pregnant and Lactating Women in Zaria, Nigeria

Figures 4.15, 4.16, 4.17, 4.18, 4.19 and 4.20 show the concentration of calcium, phosphate, vitamin D, PTH and calcitonin in the different ethnic groups. Calcium concentration was highest among the other ethnic groups and lowest among the Hausas. Phosphate was highest among the Ibos and lowest among the Hausas. Among the Yorubas, vitamin D concentration was highest and lowest among the Ibos. Serum PTH concentration was highest among the Yorubas and lowest among the Ibos. Calcitonin concentration was highest among the Hausas and lowest among the Yorubas. However, these differences were not significant.

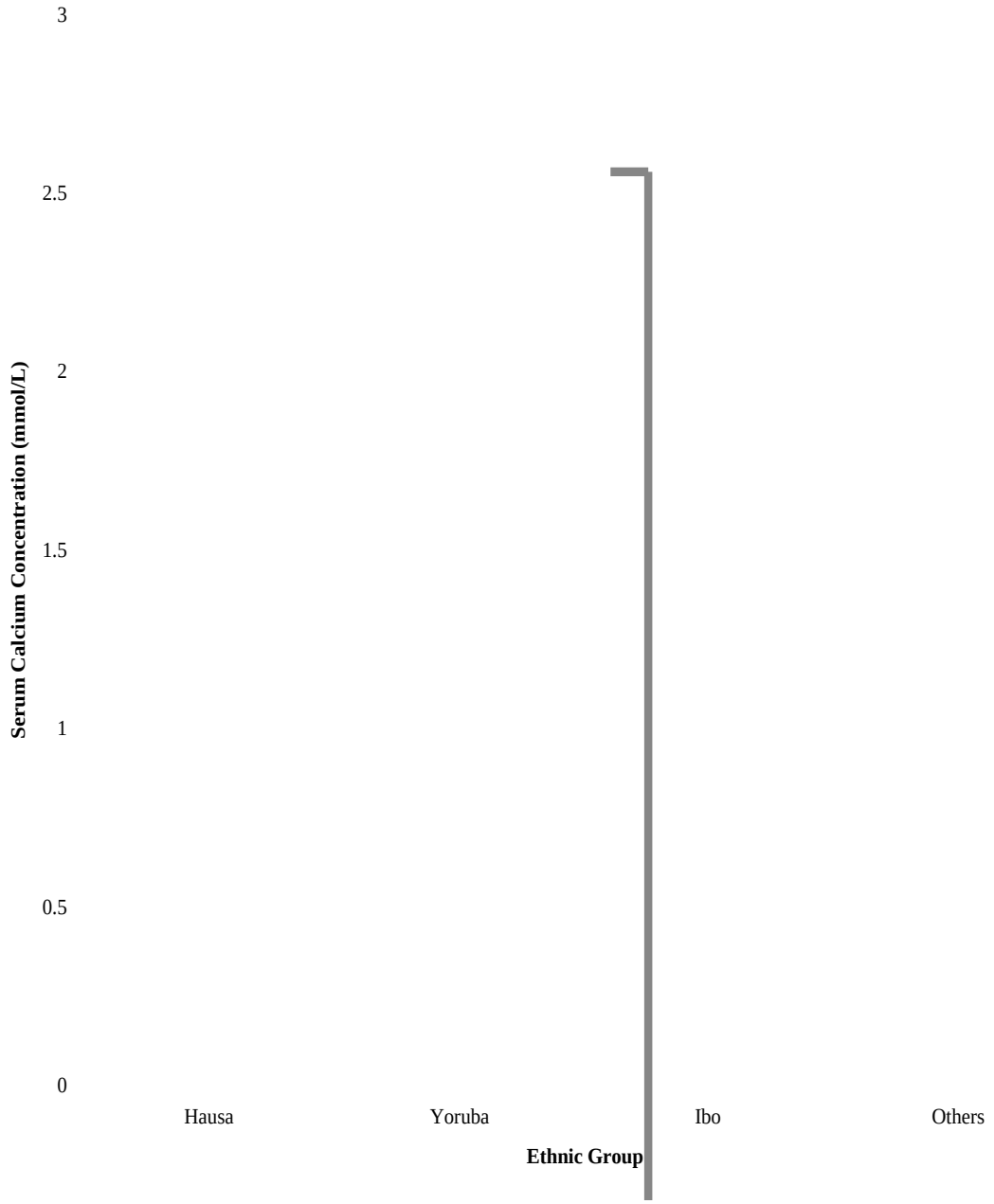


Figure 4.16: Influence of ethnicity on serum concentration of calcium in control, pregnant and lactating women in Zaria, Nigeria

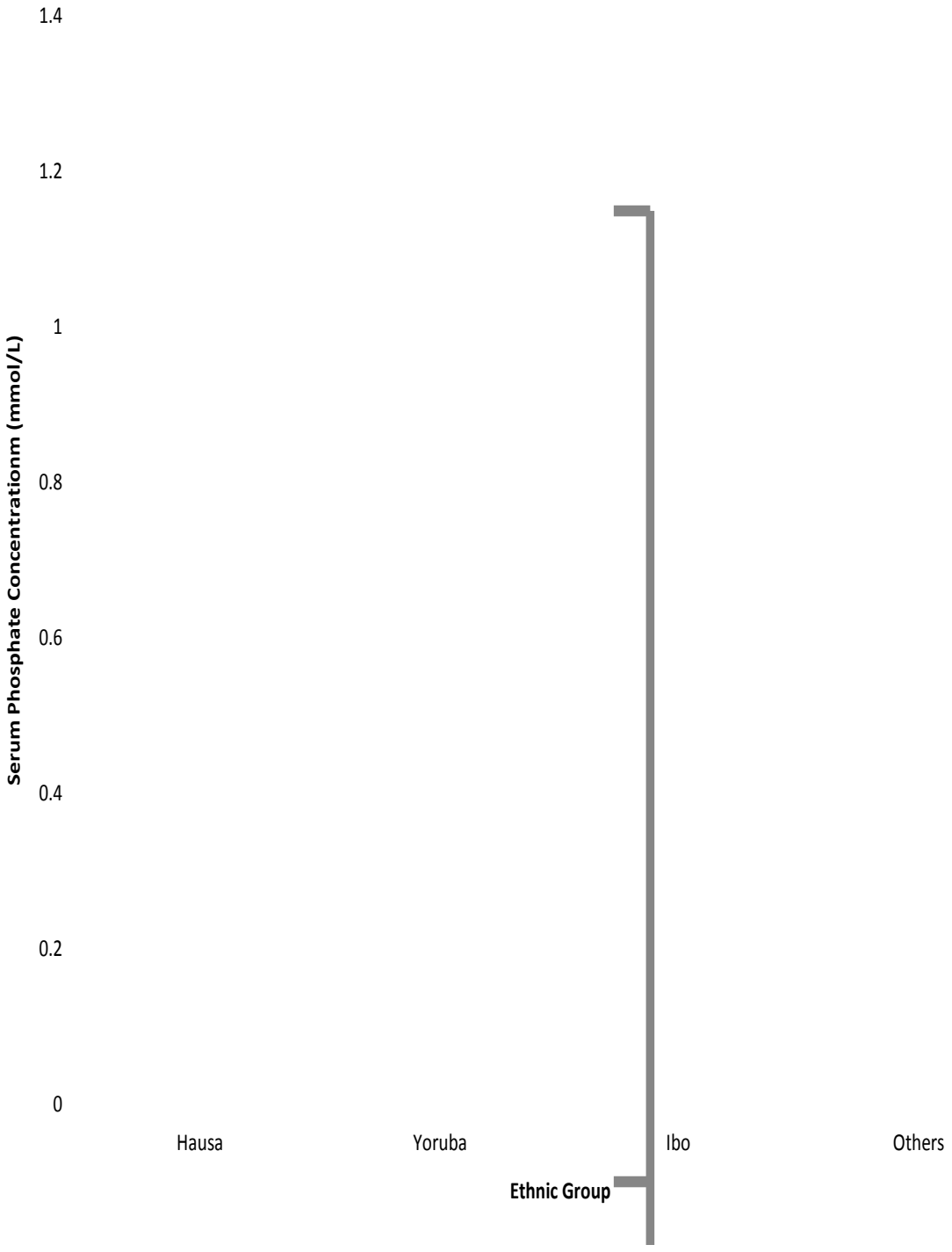


Figure 4.17: Influence of ethnicity on serum concentration of phosphate in control, pregnant and lactating women in Zaria, Nigeria

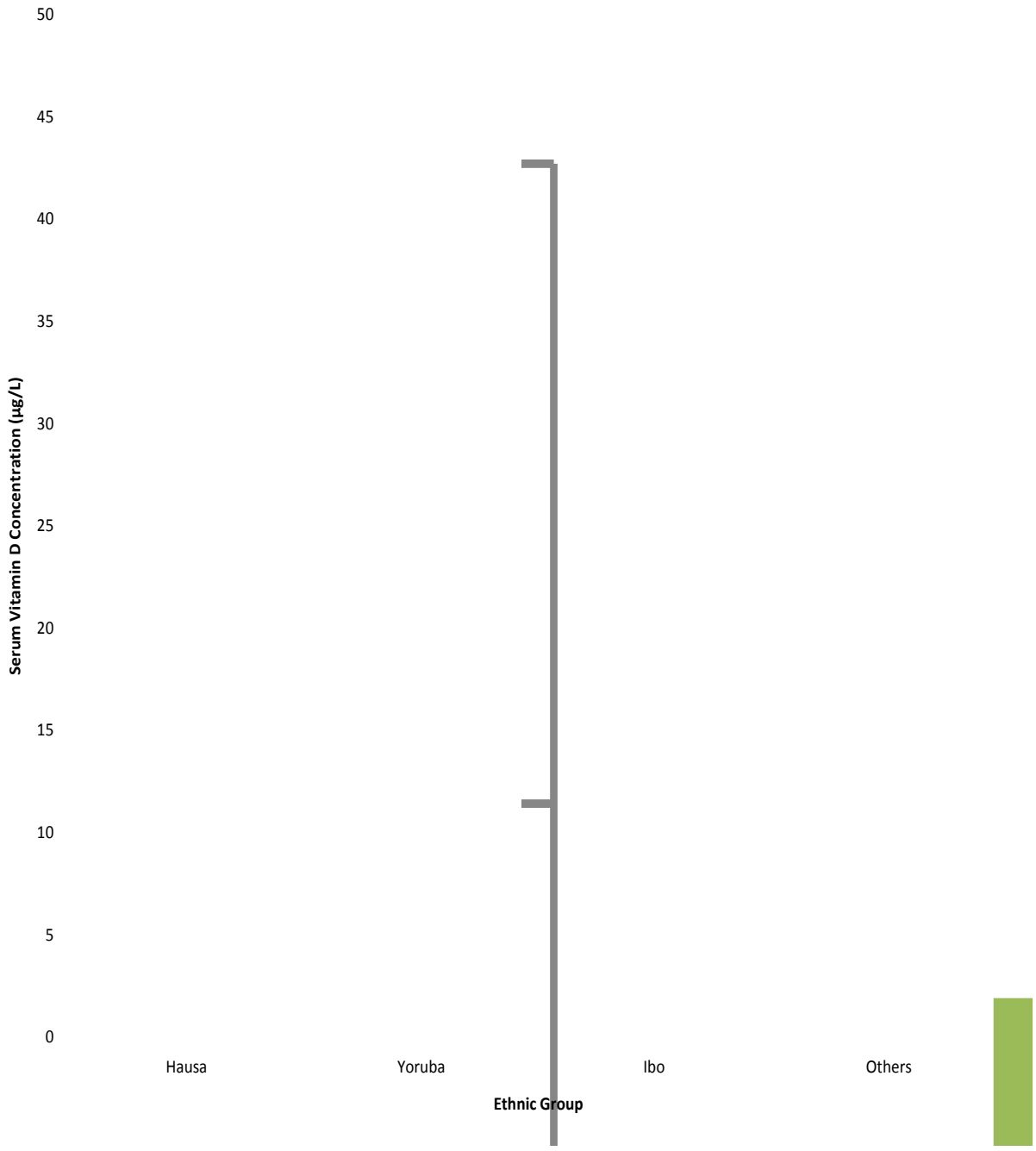


Figure 4.18: Influence of ethnicity on serum concentration of vitamin D in control, pregnant and lactating women in Zaria, Nigeria.

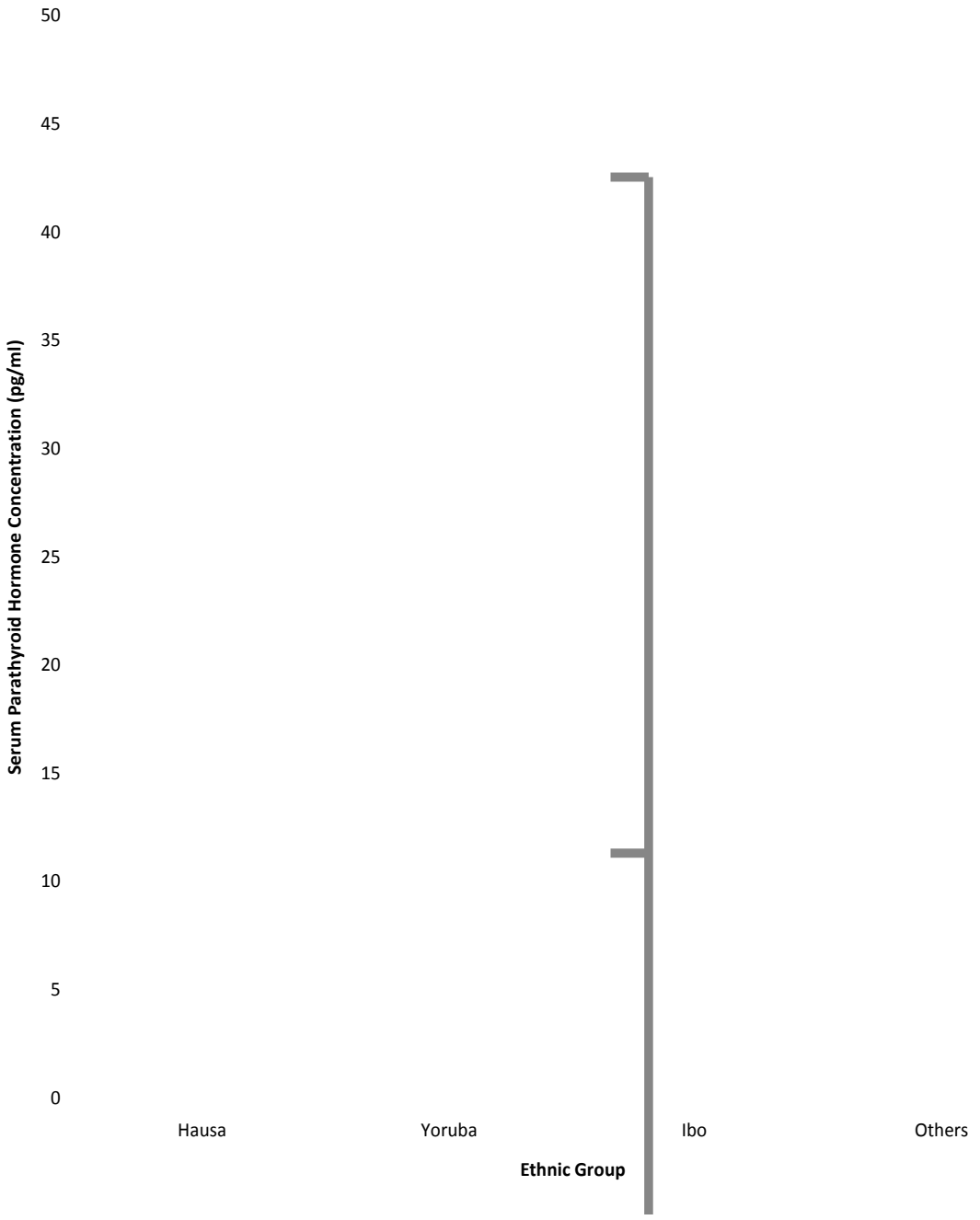


Figure 4.19: Influence of ethnicity on serum concentration of parathyroid hormone in control, pregnant and lactating women in Zaria, Nigeria.

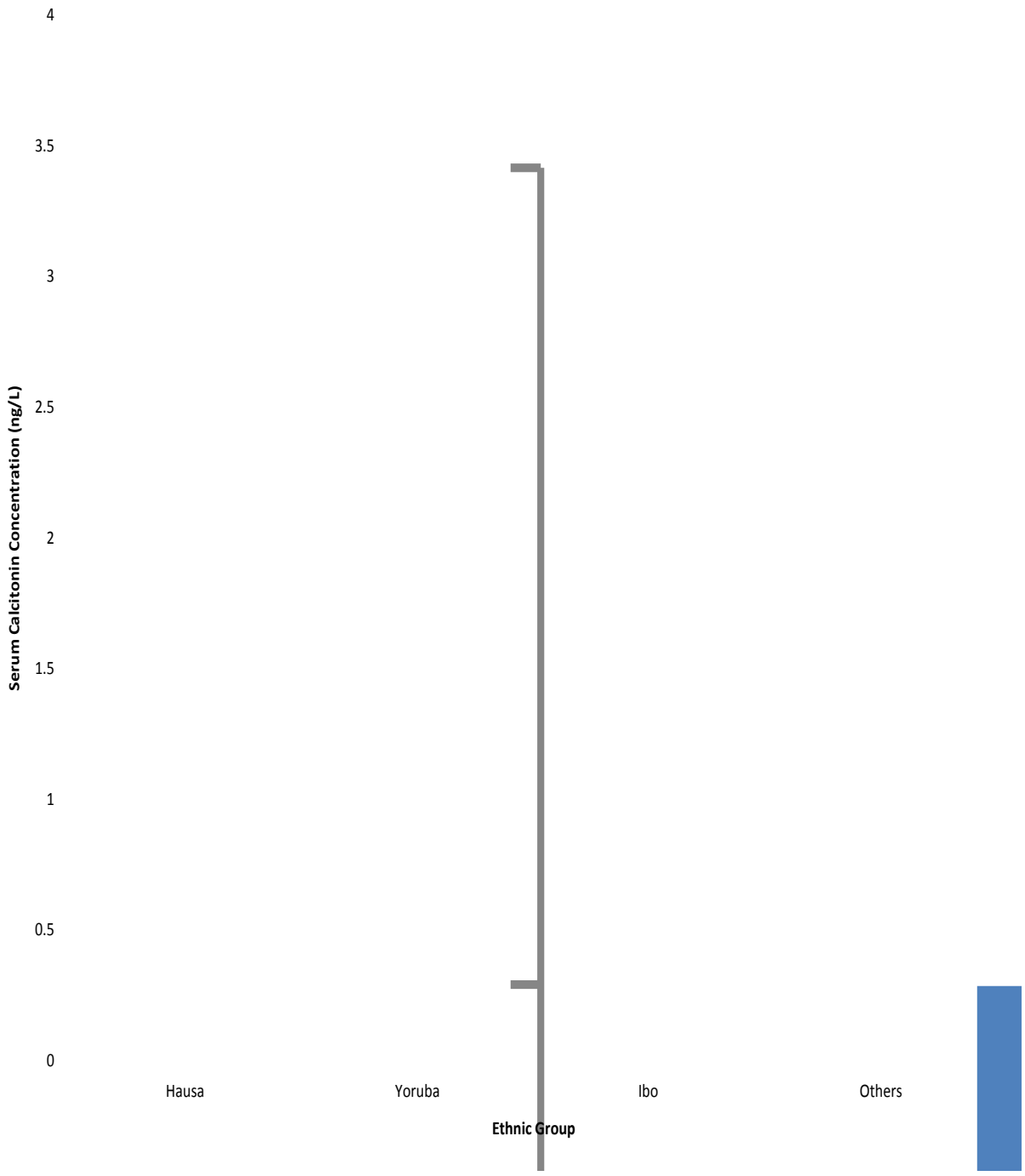


Figure 4.20: Influence of ethnicity on serum concentration of calcitonin in control, pregnant and lactating women in Zaria, Nigeria.

4.17 Influence of Ethnicity on Serum Concentration of Biomarkers of Oxidative Stress in Control, Pregnant and Lactating Women in Zaria, Nigeria

Figures 4.20, 4.21, 4.22, 4.23 show the antioxidant levels in the different ethnic groups. Serum catalase concentration was highest among the Yorubas and lowest in the other ethnic groups. Serum SOD was highest among the Ibos and lowest among the Yorubas. Serum GPx was highest in the Hausa ethnic group and lowest among the other ethnic groups. Serum MDA was highest in Yorubas and lowest in the other ethnic groups. However, the differences were not statistically significant ($P > 0.05$).

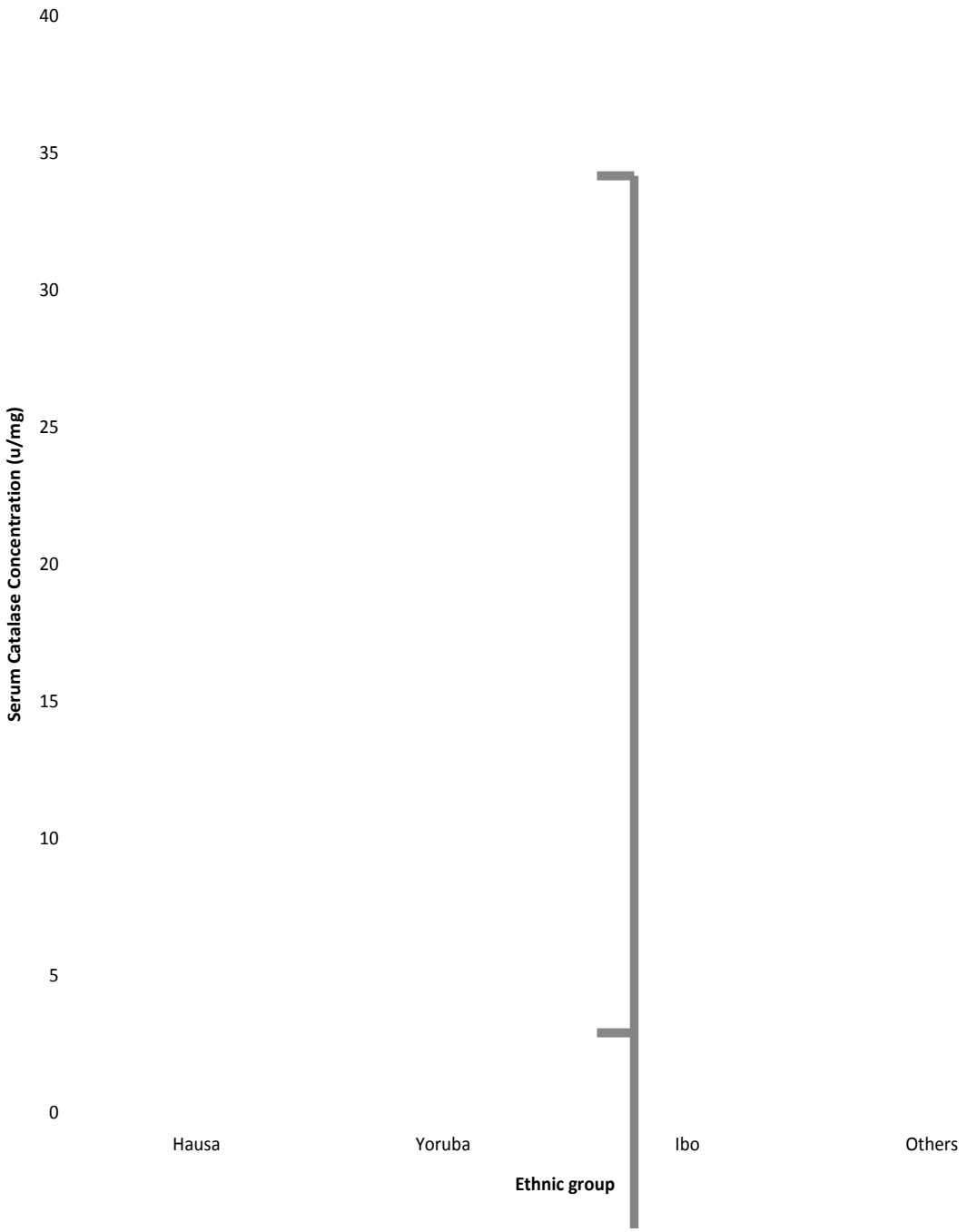


Figure 4.21: Influence of Ethnicity on serum concentration of catalase in control, pregnant and lactating women in Zaria, Nigeria

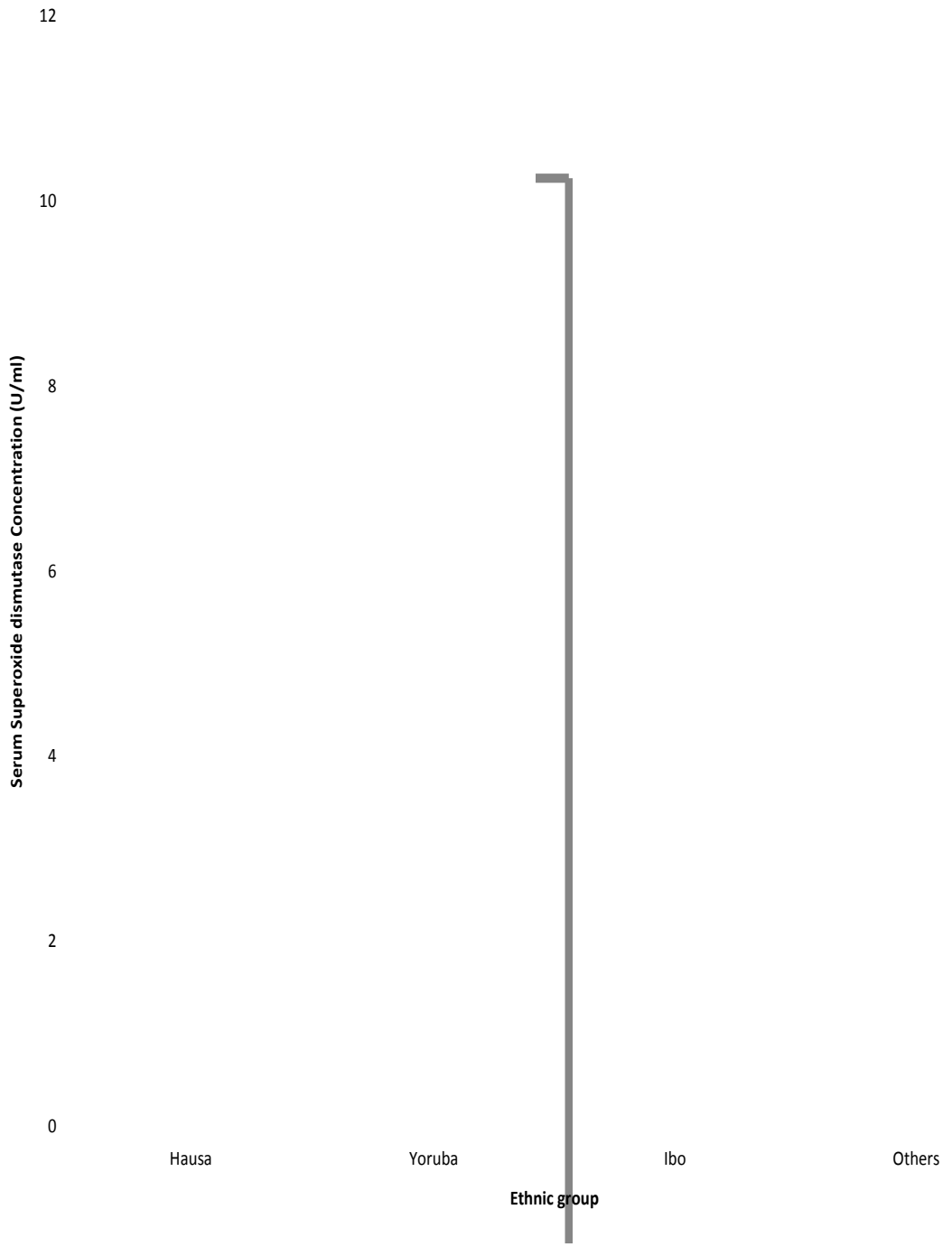


Figure 4.22: Influence of ethnicity on serum concentration of superoxide dismutase in control, pregnant and lactating women in Zaria, Nigeria

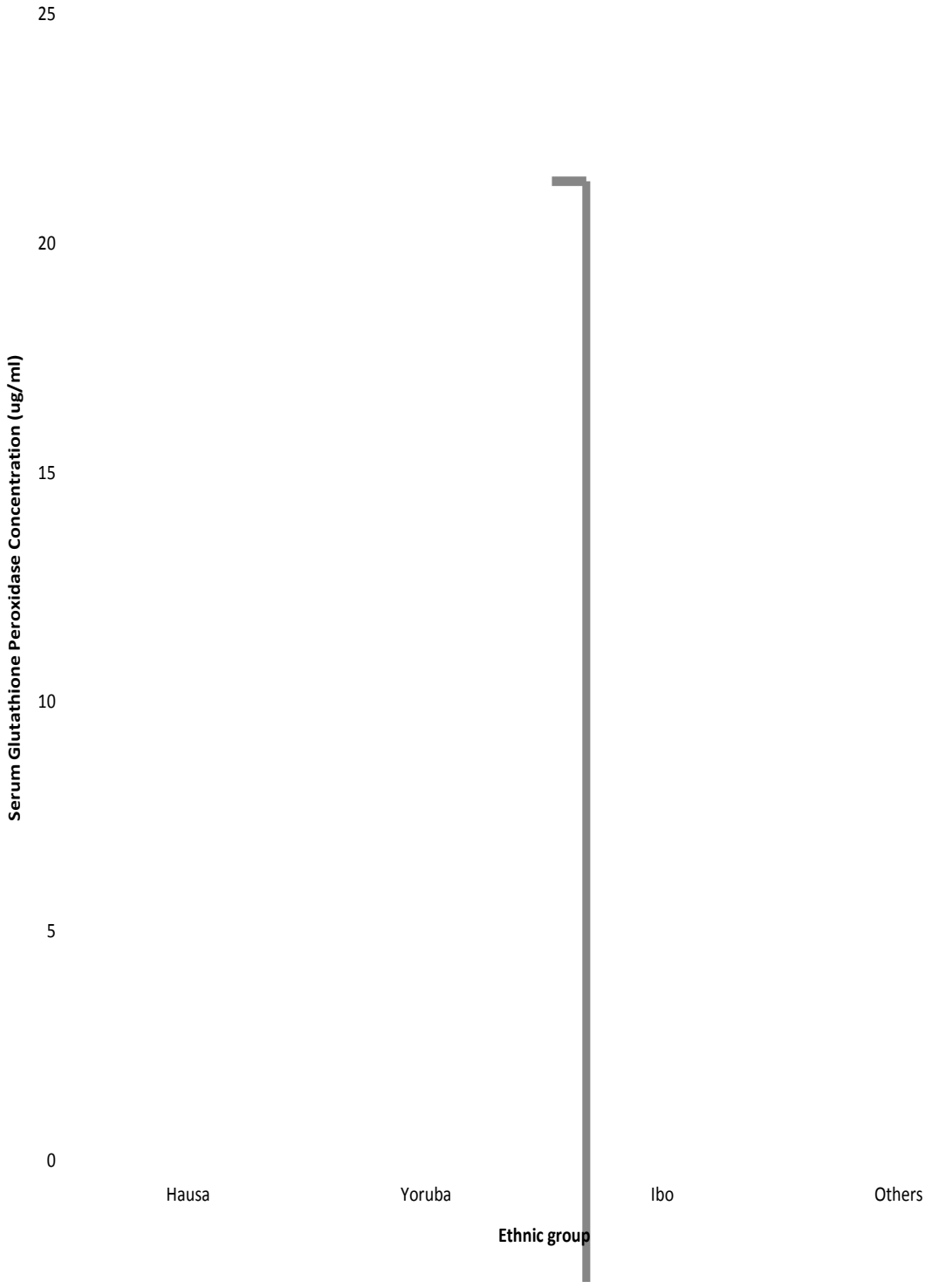


Figure 4.23: Influence of ethnicity on serum concentration of glutathione peroxidase in control, pregnant and lactating women in Zaria, Nigeria.

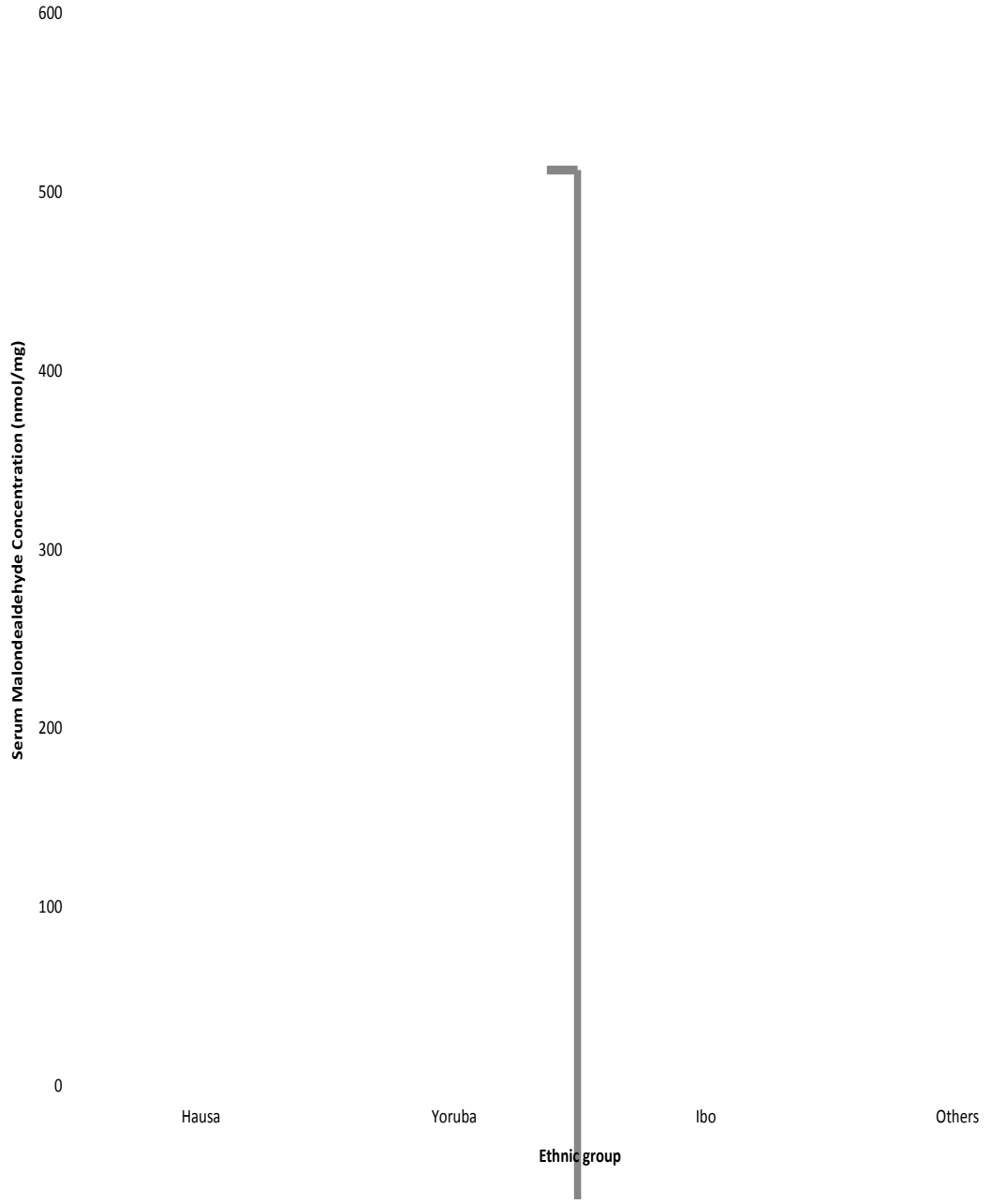


Figure 4.24: Influence of ethnicity on serum concentration of malondialdehyde in control, pregnant and lactating women in Zaria, Nigeria.

4.18 Influence of Occupation on Serum Concentration of Calcium, Phosphate, Parathyroid Hormone and Calcitonin in Control, Pregnant and Lactating Women

Figures 4.24, 4.25, 4.26, 4.27, 4.28 show calcium, phosphate, vitamin D, parathyroid hormone and calcitonin concentrations in women according to different occupations. Calcium concentration in those who do hand-work was highest and lowest in the civil servants. Phosphate was highest in the civil servants and lowest in the handwork group. The vitamin D concentration was highest in the housewife group and lowest in the civil servant group. Serum PTH concentration was highest among those who do handwork and lowest among the civil servants. Among the housewives, calcitonin concentration was the highest and lowest among those who do handwork. However, these differences were not significant.

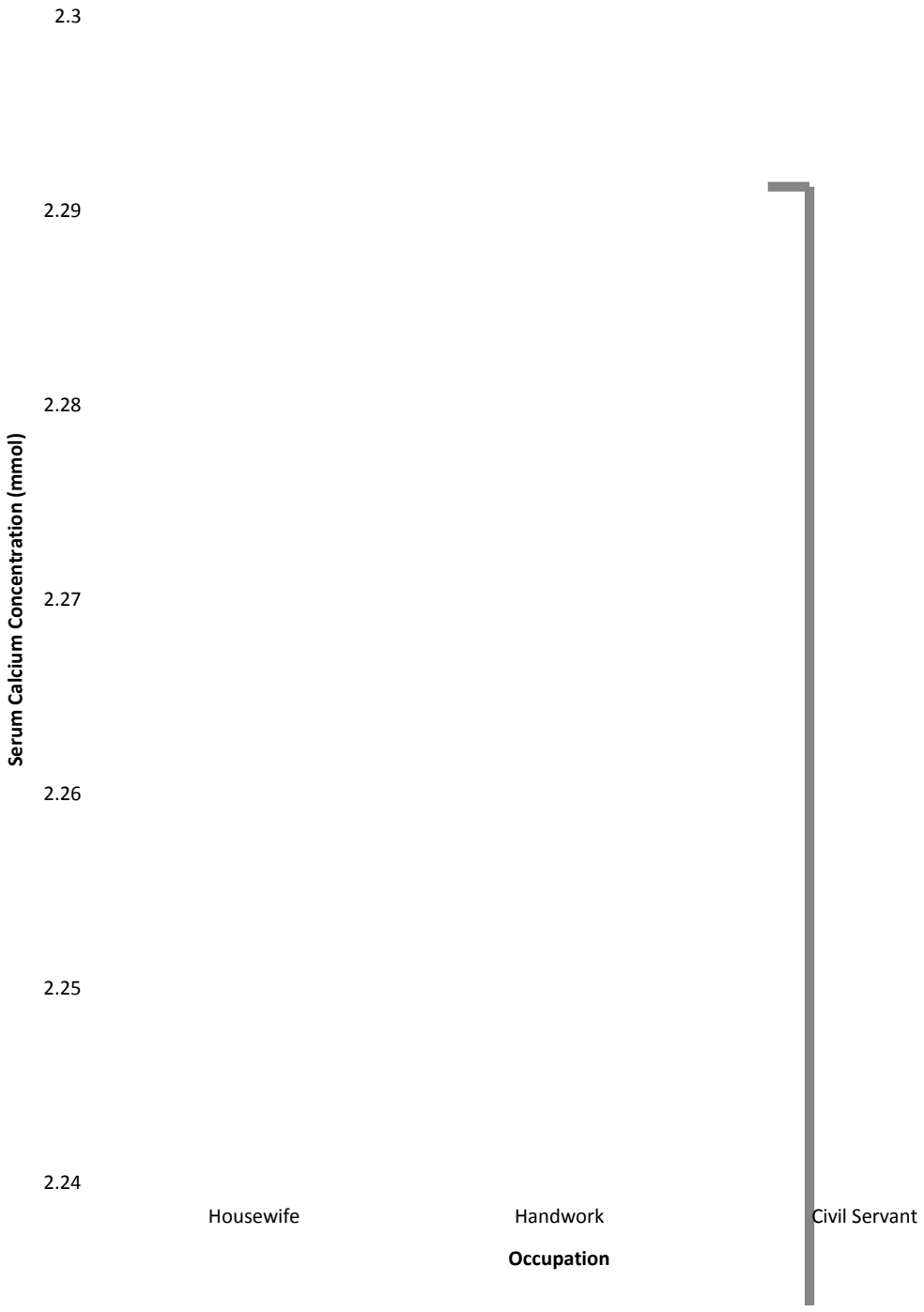


Figure 4.25: Influence of occupation on serum concentration of calcium in control, pregnant and lactating women in Zaria, Nigeria.

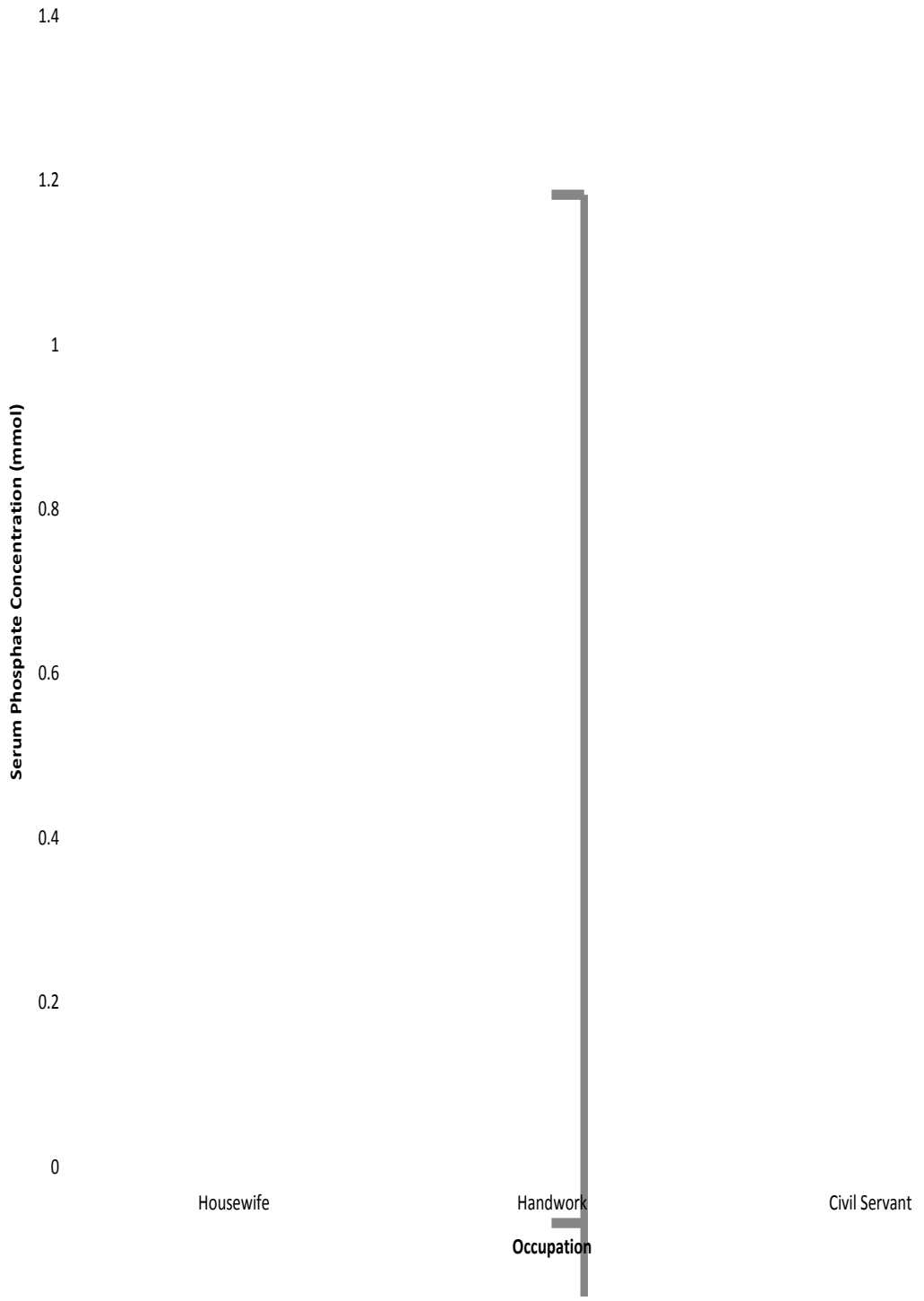


Figure 4.26: Influence of occupation on serum concentration of phosphate in control, pregnant and lactating women in Zaria, Nigeria.

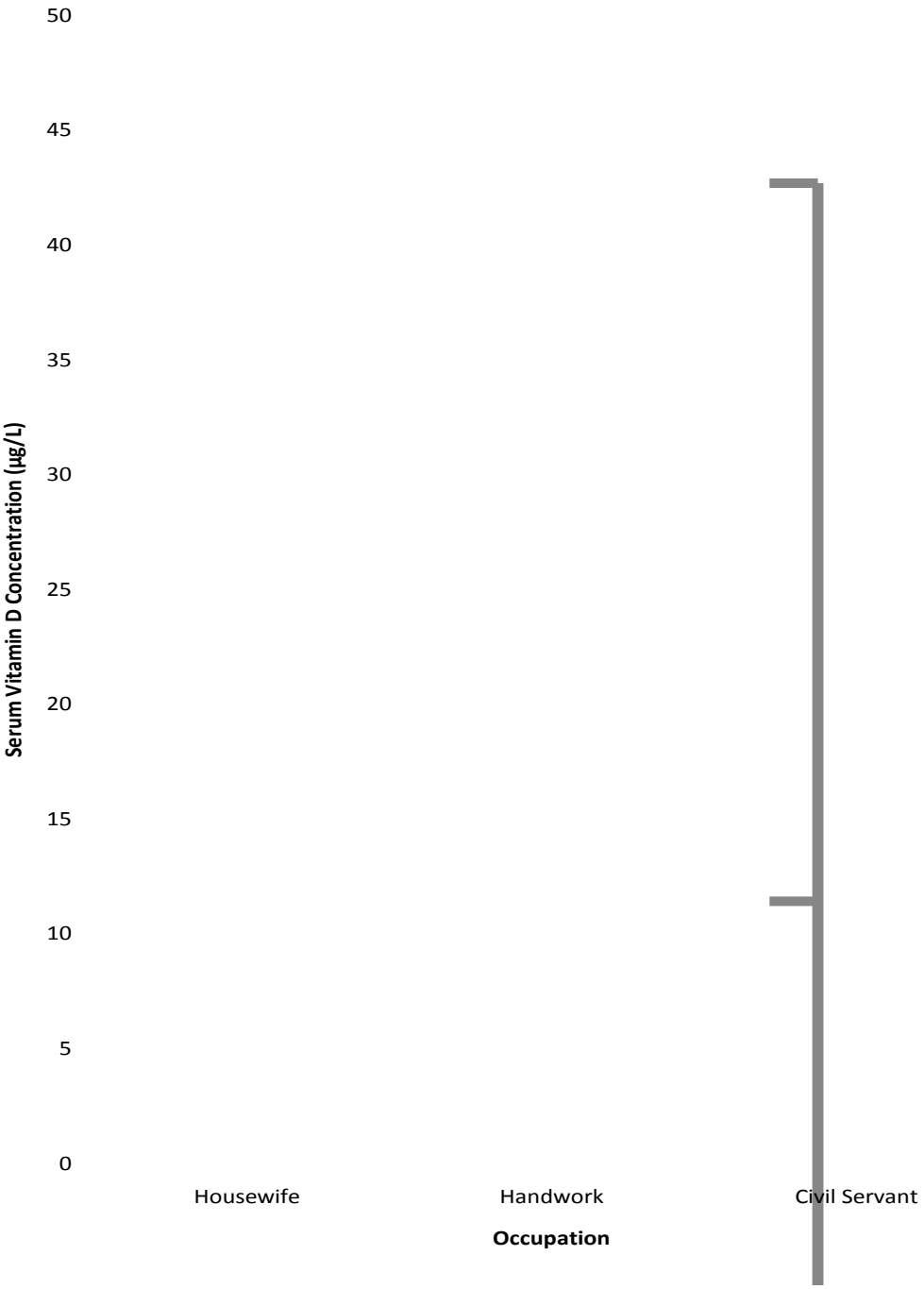


Figure 4.27: Influence of occupation on serum concentration of vitamin D in control, pregnant and lactating women in Zaria, Nigeria.

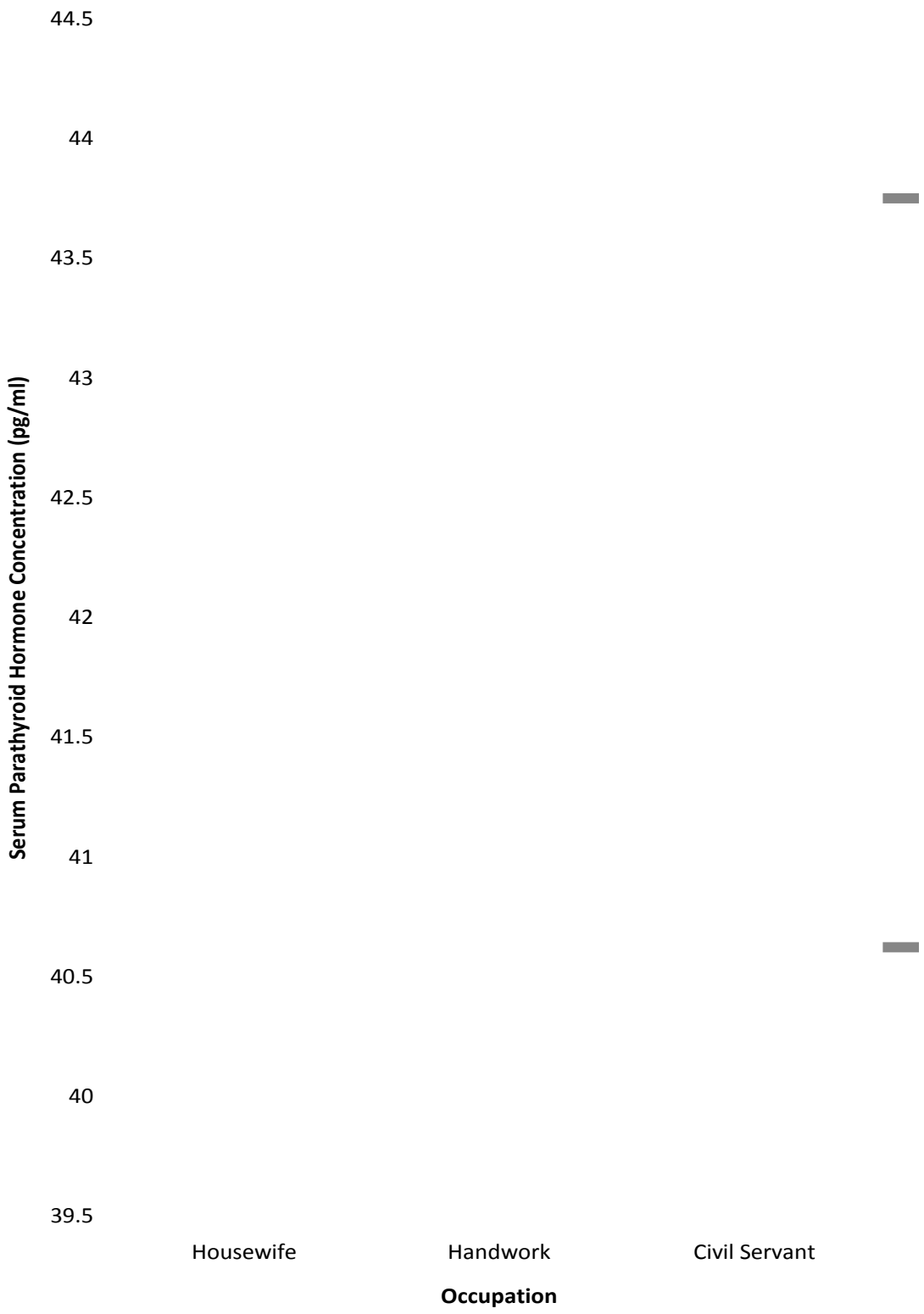


Figure 4.28: Influence of occupation on serum concentration of parathyroid hormone in control, pregnant and lactating women in Zaria, Nigeria.

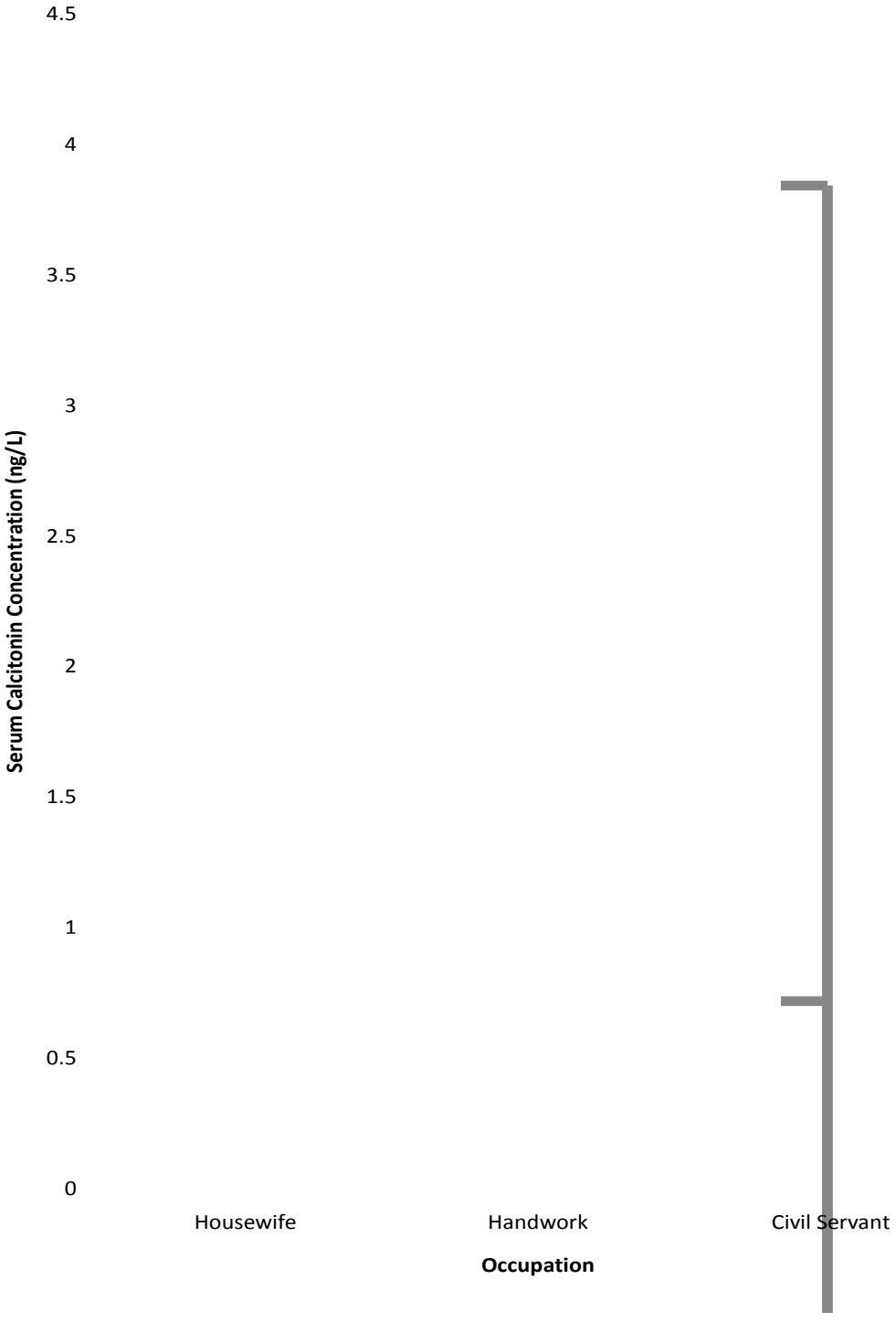


Figure 4.29: Influence of occupation on serum concentration of calcitonin in control, pregnant and lactating women in Zaria, Nigeria.

4.19 Influence of Occupation on Serum Concentration of Biomarkers of Oxidative Stress in Control, Pregnant and Lactating Women in Zaria, Nigeria

Figure 4.29 shows the antioxidant levels among the different occupational groups. Among the housewives, catalase was highest but lowest among those who do handwork. Serum SOD was highest among the civil servants and lowest among those who do handwork. Serum GSH was highest among the civil servants and lowest among the housewives. Serum MDA was highest among the housewives and lowest among those who do handwork. However, the differences were not significant ($P > 0.05$).

4.20 Influence of Body Mass Index on Serum Concentration of Calcium, Phosphate, Parathyroid Hormone and Calcitonin in Control, Pregnant and Lactating Women

Figure 4.30 shows serum concentration of calcium, phosphate, vitamin D, PTH and calcitonin in relation to BMI category. The overweight group had the highest calcium concentration, while lowest calcium level was in those who were underweight. $P > 0.05$

4.21 Influence of Location of Sampling on Serum Concentration of Calcium, Phosphate, Parathyroid Hormone and Calcitonin in Control, Pregnant and Lactating Women

Table 4.31 shows the distribution of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin between those who live around Ahmadu Bello University (ABU) environment and Tudun Wada (T/Wada) environment. It showed no significance.

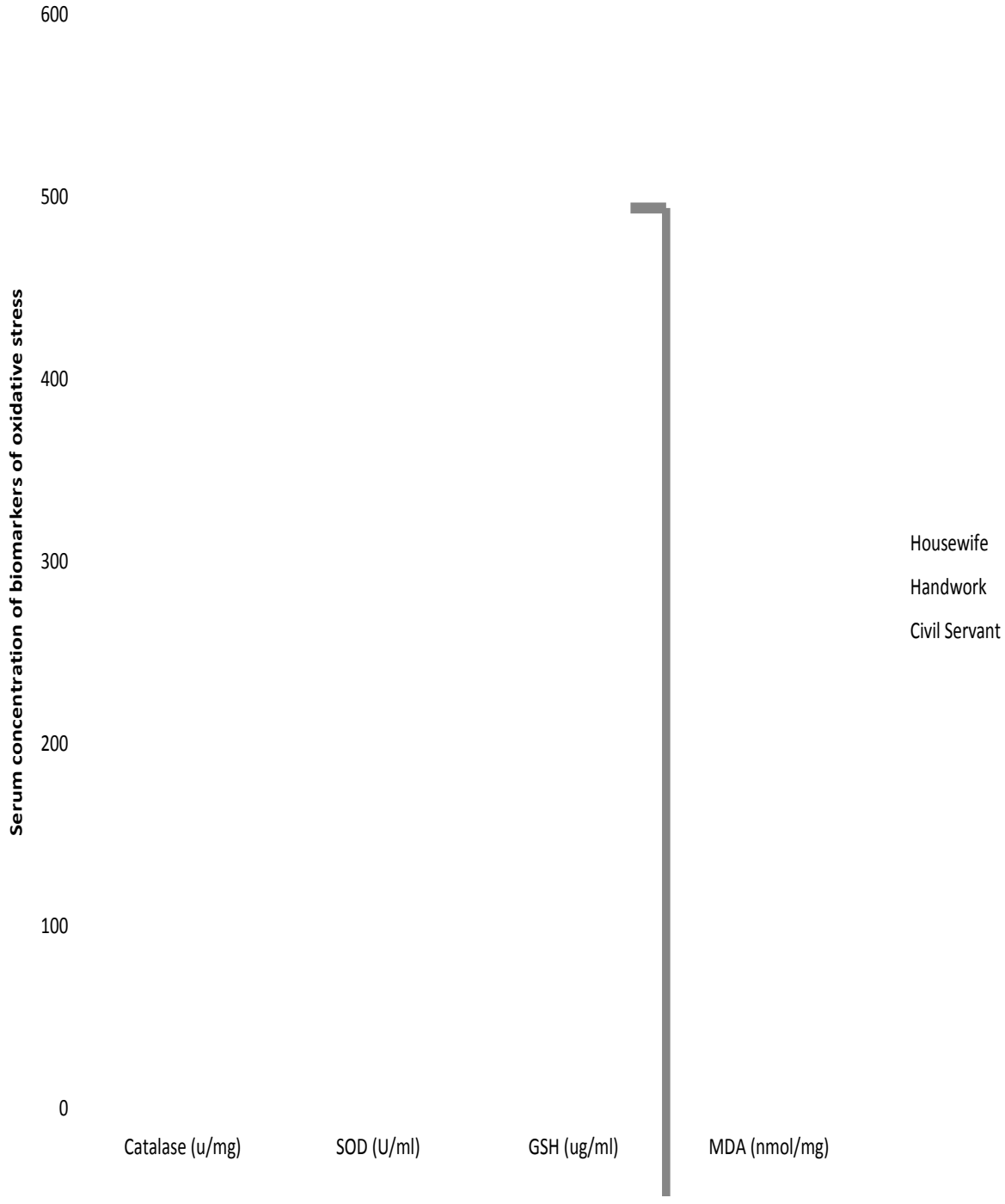


Figure 4.30: Mean (± SD) serum concentration of oxidative stress biomarkers among different occupational groups

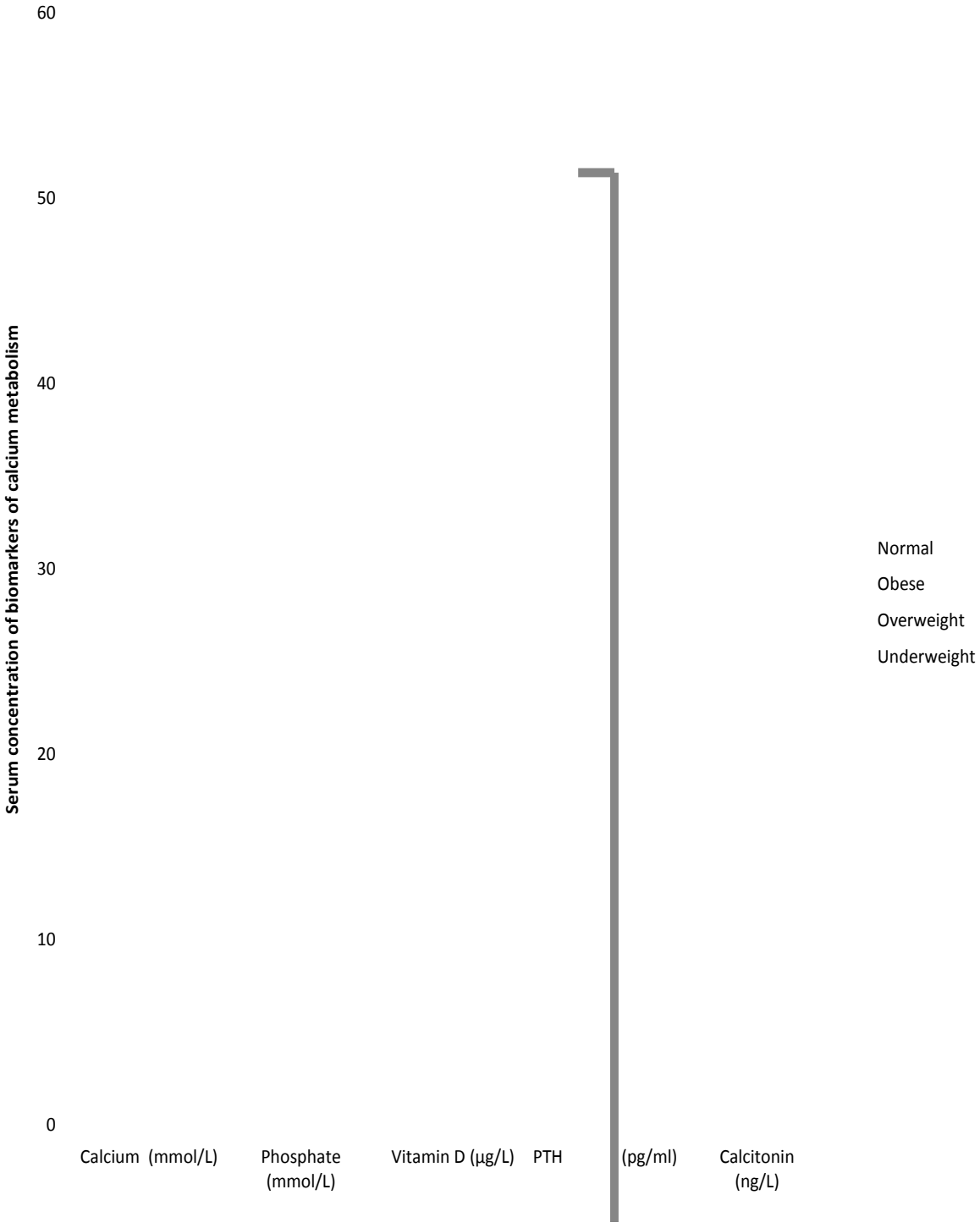


Figure 4.31: Mean (± SD) serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin concentration in relation to body mass index category

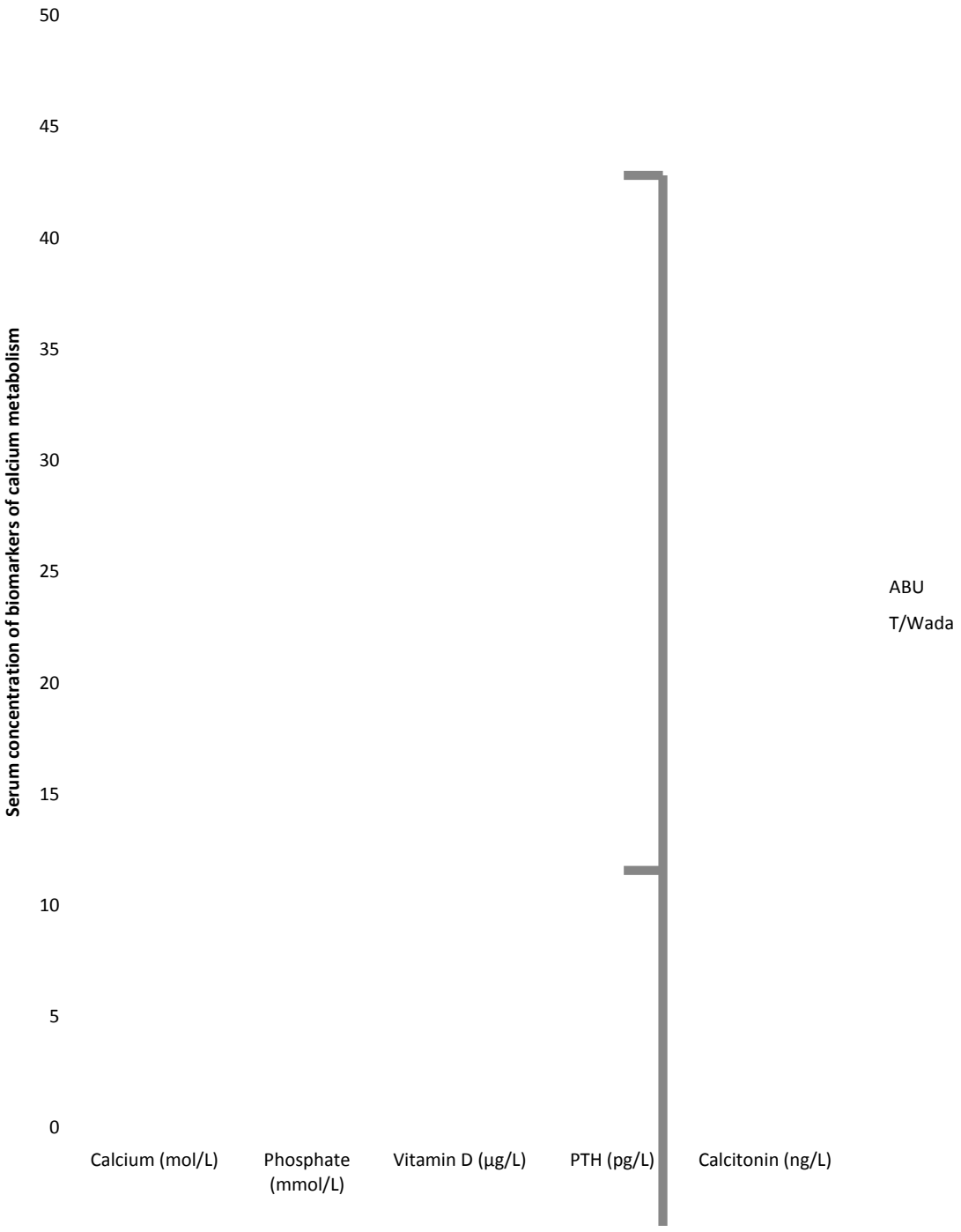


Figure 4.32: Influence of location of sampling on serum concentration calcium, phosphate, vitamin D, parathyroid hormone and calcitonin

CHAPTER FIVE

5.0 DISCUSSION

This study was conducted to evaluate the serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin and to determine if there is any association between these calcium and phosphate regulating hormones and the biomarkers of oxidative stress in pregnant and lactating women in Zaria.

Serum calcium has been reported to decrease during late pregnancy in rats, guinea pigs, and indeed humans (Dubreuil *et al*., 2005). It has commonly been measured without correcting for any change in the protein or albumin concentrations; even though a progressive 10–25% fall in protein and albumin has been found in pregnant rats, guinea pigs, and sheep (Kovacs, 2016). A decline in serum albumin triggers a decrease in serum calcium. However, in this study, ionized calcium was measured, since it is the physiologically significant fraction of extracellular calcium. In humans, the first studies in pregnant women found a progressive 5–10% fall in serum calcium in which the mean value dropped below the reference range, thus suggesting biochemical hypocalcaemia (Kovacs, 2016).

The results of the present study did not show any significant difference in serum calcium concentration between the non – pregnant women (control) and first trimester of pregnancy. This might probably be due to decreased fetal demand for calcium during the period. Also, there was a drop in serum calcium by the third trimester and during lactation which were statistically significant, when compared with the control. This is because pregnancy and lactation cause a substantial increase in demand for calcium. Thus, increased intestinal calcium absorption, which more than doubles during pregnancy. Maternal skeleton resorption also helps to provide most of the

calcium content of breast milk during lactation (Kovacs, 2008). These findings agree with what had previously been reported in animal models and human foetus. That is, calcium is removed from the maternal circulation in sufficient amounts to provoke maternal hypocalcaemia and secondary hyperparathyroidism. Some studies have shown that ionized calcium does not change during human pregnancy (Seely *et al*., 1997). However in this study, there was a decrease in the concentration of ionized calcium by the third trimester and during lactation. This finding also agrees with that of Okonofua *et al.*, 1986 who reported significantly lower serum calcium levels in pregnant purdah clad women.

There was a very low value of serum calcium concentration recorded in one pregnant women in the present study. This very low value may have been as a result of low calcium intake of less than 500 mg/day. However, it is recognized that seemingly normal serum calcium levels might not necessarily be interpreted as an outcome of adequate calcium intake but rather as a result of decreased calcium metabolism during periods of low intake. This finding was supported by the result of an earlier study on the food intake of pregnant and lactating women in Plateau State, Nigeria (Madukosiri *et al*., 2011).

In this study, more women were hypercalcaemic. The hypercalcaemia might have been as a result of calcium rich diets, such as nuts and soya bean cake supplements (tofu, *wara*) (common in Zaria and environs) which the pregnant women might have been consuming. That is, assuming that they consume adequate amounts of calcium (1,000 mg/day) from regular diet (Hacker-Thompson *et al.,* 2012). Taking calcium supplements could cause raised concentrations of serum calcium. The results of this study also showed a significant negative correlation between gestational age and serum

calcium. Similarly, Benali and Demouche (2014) observed a high prevalence (70.55%) of hypocalcaemia among pregnant women in Algeria. In another study by Ainy *et al*. (2006) in Iran, it was discovered that 19% of 48 pregnant women studied had hypocalcaemia, while Kumar *et al*. (2010) reported that approximately two thirds of pregnant women in India were hypocalcaemic though, none of the women had any manifest symptoms.

In contrast however, another study by Olatunbosun *et al.,* (1975) observed no hypocalcaemia during pregnancy. The latter study reported that the reasons for infrequency of osteomalacia and for the relatively high serum concentration of calcium in pregnancy in our environment as at that time, were unclear. However in another study by Eller-Vainicher *et al*. (2012), a patient who had hypocalcaemia due to increased plasma concentration of PTHrP, was relieved of the symptoms after delivery. Apart from increased PTHrP, other causes of hypercalcaemia could be as a result of renal insufficiency and metabolic alkalosis that result from the overconsumption of calcium containing products (Kolnick *et al*., 2011).

In the present study there was a rise in serum concentration of phosphate from the 1st to the 3rd trimester; though this rise was not statistically significant. This is in agreement with the study by Kametas *et al.* (2003) which showed that serum phosphate levels are within the non-pregnant range; possibly because of increased renal reabsorption and skeletal resorption during pregnancy and lactation. Similarly, phosphorus is involved in the intermediary metabolism of carbohydrates and is a component of other physiologically important substances such as organic phosphate esters, phospholipids, nucleic acids and nucleotides. Thus, the observed increase in serum phosphate level is linked to a possible increase in macromolecular metabolism during pregnancy

(Kametas *et al*., 2003). It could also be due to the relative decrease in serum calcium, because the ratio of calcium to phosphate is 2:1 in the blood. Therefore, when calcium decreases; phosphate levels will automatically increase. Thus, the increase in phosphate concentration may be apparent; not real.

This is also in agreement with most studies on serum phosphate concentration which shows no significant change during the course of pregnancy (Cross *et al.,* 1995; Møller *et al.,* 2013). Although, there are other confounding reports such as that of Sanchez *et al.* 1997 who reported that all the women in their study had marked hypophosphataemia, though their sample size was relatively small.

In another study by Ikekpeazu *et al.* (2010) serum inorganic phosphate levels showed significant decrease in the second and third trimesters as compared to non-pregnant, non-lactating group. Similarly, Okonofua *et al.* (1986) also reported lower phosphate concentration in purdah clad women. The population of pregnant women in the present study showed a prevalence of 1.76% hypophosphataemia and none was observed to have hyperphosphataemia.

In the present study, vitamin D concentration was highest in the 2nd trimester. But the 3rd trimester showed a lower vitamin D concentration than the 1st trimester. Although, the difference was not significant, it may be physiologically significant. The higher vitamin D levels observed in this study during the 2nd trimester of pregnancy is in accordance with the finding of Møller *et al*., 2013. The synthesis of vitamin D in the skin, which depends on adequate exposure to ultraviolet sunlight, is affected by skin pigmentation levels, age, obesity, clothing, and use of sunscreen products, latitude, season and time of day (Lundqvist *et al.,* 2016). Since Nigeria is in Latitude 9.0820° N,

8.6753° E, where synthesis of vitamin D is high and and coupled with the season of the study (August – September 2015), it can be concluded that the conditions were favourable to vitamin D synthesis in the body and the higher levels observed in the second trimester maybe due to a negative feedback stimulation from decreased calcium levels as at that period.

Also the rise in serum vitamin D could be due to upregulation in the synthesis of vitamin D and the activity of gene Cyp27b1, which can be found in the maternal kidneys, maternal decidua, placenta, and fetus (Kovacs, 2016). All these factors could contribute to a rise in vitamin D concentration by the 2nd trimester of pregnancy. The hypovitaminosis D observed as compared to the control in this study was 6.19%. This is in contrast to the 29% vitamin D deficiency reported by Gbadegesin *et al.* (2017) in a study of pregnant women in Lagos, Nigeria.

In this study, hypervitaminosis D was 21%. There was strong negative correlation between vitamin D and calcium and vitamin D and phosphate, which was statistically significant. This suggests that as vitamin D level increases, calcium and phosphate concentrations were decreasing. Kirby *et al.,* 2013, using rats and mice, demonstrated that serum vitamin D concentrations could increase up to seven times during the course of a normal pregnancy and this corresponds to the time of rapid foetal mineral accretion (in the third trimester of pregnancy) which could lead to reduced serum calcium levels. This is consistent with the increased serum vitamin D concentration observed in this study. It has also been suggested that the maternal kidneys are a probable site for increased vitamin D production (Kovacs and Ralston,2015), as a result of an upregulated synthesis, also Yates *et al.,* 2017 and Nguyen *et al.,* 2015 demonstrated that vitamin D is also produced by the placenta. Other sources of

increased serum vitamin D concentration could come from the diet and increased intestinal absorption or by reducing excretion via the kidney or gastrointestinal tract (Fukumoto and Shimizu, 2011). While, the pregnant woman's body provides 25 to 30 g of calcium to support the developing foetal skeleton (Holt, 2013). It could then be extrapolated that the negative correlation between vitamin D and calcium occurred because, for growth to take place, the essential elements such as sodium, potassium and calcium are usually diverted to the site of growth and synthesis; and as such, due to the proliferation of the lactiferous ducts and lobules of the breasts and due to foetal calcium accretion (especially during the 3rd trimester of pregnancy and the lactation period), more calcium is required during this phase. As a result of this increase in demand, there is a negative feedback triggered causing a decrease in the blood serum concentration of calcium and the subsequent increase in vitamin D synthesis, intestinal absorption and reduced renal excretion.

There was also a significant positive correlation between vitamin D and parathyroid hormone. In contrast, Reddy *et al.* (1983) reported that increased vitamin D concentration did not correlate with parathyroid hormone concentration. Okonofua *et al*. (1986) also reported a significant decrease in serum concentration of vitamin D in purdah clad pregnant women than their non-purdah counterparts in Southwest Nigeria.

In this study, parathyroid hormone levels were higher in the 1st trimester and 2nd trimester, and lower in the 3rd trimester. This finding is in agreement with earlier studies (Madar *et al.,* 2009; Marwaha *et al.,* 2011; Roth *et al.,* 2013 and Schoenmakers *et al.,* 2013). In this study, there was a prevalence of 0.88% hypoparathyroidism and 7.97% hyperparathyroidism. Parathyroid hormone showed a significant negative correlation with calcium. This finding corroborates with the report of Gillies *et al.*

(2015) who reported that high calcium in the diet caused a lower serum PTH concentration. Some animal models have shown that pregnant mice have very low PTH levels (Kirby *et al*., 2013), these maybe as a result of negative feedback mechanisms. Other human studies such as the cross-sectional analyses of European and North American women who were on a sufficient calcium diet, showed that PTH is usually suppressed to the lesser end of the average range during all three trimesters of pregnancy. With the use of contemporary assays, it has also been confirmed that suppression of PTH occurs in pregnant women of African and Asian extraction (Ainy *et* al., 2006; Shao *et al.*, 2012) as a result of negative feedback effect. In these regions, many cohort and longitudinal studies have discovered that PTH did not decrease during pregnancy, while some studies reported a rise higher than normal (Marwaha *et al.*, 2011; Schoenmakers *et al.*, 2013; Roth *et al.*, 2013).

Holt, 2013 also suggested that decline in PTH during pregnancy could be as a consequence of direct inhibition of PTH production either by previously high concentrations of 1,25-dihydroxyvitamin D or by augmented intestinal absorption of calcium. This result substantiates that PTH concentration varies during pregnancy along the different trimesters, as can be seen in the negative correlation between serum PTH and calcium concentrations. Though, this correlation may not be statistically significant, it may have biological relevance. Since only small quantities (pg to ng) of hormones are required to cause significant physiological changes in target tissues.

In this study, serum calcitonin levels were increased in the 2nd trimester, but decreased by the 3rd trimester. There was also a slight decrease in serum calcitonin concentration in the lactating group. There was no hypocalcitoninaemia observed in this study, but there was a 1.76% prevalence of hypercalcitoninaemia. This finding supports the report

of Ardawi *et al.*, 1997. However, it contradicts the findings of Møller *et al.* (2013) who found no change in the level of calcitonin; and Silva *et al.* (1981), who found decreased levels of calcitonin during the three trimesters of pregnancy.

The increase in calcitonin level during the 2nd trimester may be attributed to increased production by the C-cells of the thyroid gland and additional sources of expression that may contribute to the circulating level of calcitonin, such as mammary tissue, placenta, and pituitary tissue (Kovacs, 2016). Increased synthesis of calcitonin could reasonably explain the higher levels seen during the course of the 2nd trimester. In this study, there was a statistically significant negative correlation between calcitonin and phosphate serum concentration. In the study by Silva *et al*. (1981), they observed a similar correlation, but only in the first trimester.

The differences observed between this study and others, may be attributed to the differences in continental regions where the experiments were conducted. Some of these studies were done in the United States and other continents. The methodology used and assay methods could also account for some of the reported differences. The slight increase in serum calcitonin concentration observed in the lactating group could be attributed to its possible physiological role in protecting the maternal skeleton during pregnancy and lactation (Kovacs, 2016). Although, this concept has not been directly tested as there are no comparable human data for the surgical or genetic models of calcitonin deficiency during pregnancy.

Regression equations formulated for estimating serum calcium levels in pregnant women in all the trimesters of pregnancy and in lactating women were significant and so can be used to predict serum calcium levels during pregnancy and lactation.

Pregnant women are at an increased risk of elevated inflammation, and oxidative stress, especially during the third trimester. These conditions might result from micronutrient deficiency, increased maternal adipose tissue, and production of hormones by the placenta (Asemi *et al.,* 2016). It has also been previously reported that the influence of vitamin D on the metabolism of calcium and phosphorus is associated with some systemic diseases, and is considered an important indicator of general health (Asemi *et al.,* 2016).

The serum malondialdehyde concentration was highest in the lactating group and lowest in the control group. Gestational age correlated negatively with antioxidants, and positively with malondialdehyde but was not statistically significant. Significant positive correlation was observed between malondialdehyde and catalase; between malondialdehyde (MDA) and gluthathione peroxidase and also with phosphate. Higher MDA levels in the 2nd trimester than non-pregnant control has been reported in pregnancy due to increased generation of oxygen radicals following increased oxygen demand in pregnancy and the reduction in the activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Ugwa *et al*., 2014).

In the light of the findings in this study, it seems reasonable to speculate that synthesis of CAT may reduce oxidative stress in the uterine environment especially during the second trimester. In addition to the suggestion that CAT might come from different sources in the serum. The pregnant human myometrium might be very efficient in the elimination of MDA (Biberoglu *et al*., 2016). Thus, the higher concentration of MDA, SOD, GSH and also CAT, in the serum samples during the 2nd trimester and indeed significantly in the lactating group (SOD), may reflect the overflow of these markers

from the uterus to the circulation. It could also be that there is higher placental oxygen in the 2nd trimester than in the 1st trimester. This may possibly explain why MDA and CAT levels were observed to be low during the first trimester. A similar relationship has been previously demonstrated between the placenta and umbilical cord blood in pregnant women (Wang *et al*., 1996).

In accordance with the ischemia-reperfusion phenomenon, reoxygenation would facilitate the transfer of oxidative markers from the myometrium to the maternal circulation, not only to play a local protective role, but also as a means of responding to systemic oxidative stress (Biberoglu *et al*., 2016). An alternative explanation could be that, independent from the systemic anti-oxidant process, a well-functioning myometrial system might be active enough to rescue the pregnant myometrium from reactive oxygen species, as reflected by the values obtained in the 2nd trimester when compared with the control and the 1st trimester values obtained in this study. Additionally, the reactive oxygen species may promote local myometrial increase in oxidative stress, which are rapidly detoxified, thereby elevating oxidative markers in the circulation only slightly and for a short time; which also explains the high MDA and antioxidant levels in the circulation by the 2nd trimester observed in this study (Biberoglu *et al*., 2016).

It may well be that the increased levels of antioxidants enzymes in serum samples of pregnant women observed in this study is an indication of the effort to compensate for the elevated MDA concentration in serum, especially during the 2nd trimester.

Another finding of this study is that serum CAT was elevated during the 2nd trimester. This may be attributed to the fact that the subjects were drawn from a population of women lactating at different stages (i.e. colostrum, transitional and mature milk).

Another way to put the contradictory findings into perspective is the methodological problems in measuring free radicals and absolute levels of *in vivo* oxidative stress. In the present study, we measured four basic oxidative stress and antioxidant activity markers. Given that not all markers change in the same direction, measuring more markers makes the interpretation of the results more problematic. Moreover, to what extent the tissue-specific oxidative damage is reflected systemically and is easily measured in the serum sample is not too clear (Biberoglu *et al.*, 2016).

It can be inferred that pregnant women with increased serum levels of inflammatory biomarkers are at elevated risk of adverse pregnancy outcome, premature delivery, and disturbances of calcium homeostasis. To reduce maternal and foetal complications resulting from unfavorable metabolic profile, various strategies have been proposed such as the consumption of antioxidants, calcium and vitamin D supplementation, which help to reduce systemic inflammation and oxidative stress (Asemi *et al.*, 2016). In this study, we report the concentration of the various antioxidant enzymes and also the level of malondialdehyde in pregnant and lactating women.

The result of this study did not show any significant correlation between calcium and any of the antioxidants and malondialdehyde concentration. In a study by Fedirko *et al*., 2010, it was observed that antioxidant enzymes in humans function in combination with low-weight antioxidant compounds. Vitamin D activates the expression of antioxidant enzymes, which may not function properly in the antioxidant-depleted environment. Therefore, it is possible that calcium and vitamin D effects on the oxidative DNA are modified by the presence or absence of various pro-oxidant or antioxidant exposure. Thus, the use of calcium and Vitamin D_3 supplements may decrease oxidative DNA damage in mucosa cells (Asemi *et al.*, 2016).

This study is in agreement with this report in that there was an observed correlation between Vitamin D with SOD and GSH which are antioxidant enzymes. Similarly, in an earlier study by Ekici *et al,* (2009), increased GSH activity was seen with Vitamin D_3 supplementation in both cortex and corpus striatum in rats. Zhang *et al.,* 2016 demonstrated that vitamin D supplementation in pregnant women resulted in increased GSH levels and a significant difference in plasma MDA levels. Asemi *et al*. (2014) had previously presented data in support of this, revealing that vitamin D supplementation significantly increased GSH levels.

Lastly, some conflicting reports in the literature may be a consequence of flaws in the design of these studies, such as inclusion criteria, small study groups, calculation of sample size and methodology. In conclusion, we believe that increased generation of oxygen free radicals (oxidative stress), defense mechanisms and calcium homeostasis are closely associated. Yet, the oxidative stress in circulation must be interpreted with great caution. The increased or decreased oxidative stress marker levels in the serum samples may represent reactive changes in the context of an endothelial or inflammatory status. Thus, larger multicenter studies may be required to clarify the discrepancies seen.

All parameters compared with other factors such as age, parity, occupation, body mass index and residential location showed no statistical significance. However, these differences may have inherent biological significance if correlated with other physiological variables or when larger sample sizes are used. The significant difference in CAT levels observed when comparism was made between the ethnic groups (Hausas, Yorubas, Ibos and other ethnic groups; ($P < 0.05$) testify to these possibilities.

CHAPTER SIX

6.0 Summary, Conclusion, Recommendations and Contributions to Knowledge

6.1 Summary

This study has established that there is a very low prevalence of calcium, phosphate and vitamin D deficiency or insufficiency; even though the serum concentration of calcium decreased across the trimesters and the period of lactation. Local base line reference values for calcium (2.00 - 2.56 mmol/L), phosphate (0.78 - 1.34 mmol/L), vitamin D (40.89 - 45.93mmol/L), parathyroid hormone (23.93 – 57.65 pg/ml) and calcitonin (1.65 – 5.29 ng/ml) for pregnant women and predictive equations for serum calcium levels during the different trimesters of pregnancy and the period of lactation were formulated. Malondialdehyde (MDA) concentration increased during lactation, while superoxide dismutase and glutathione were significantly higher in the lactating group. Trimester of pregnancy influenced serum concentration of calcium but did not influence serum concentration of phosphate. Also age and body mass index did not influence serum calcium and phosphate concentrations. There was a positive correlation with vitamin D when compared with superoxide dismutase and glutathione; also, with calcium when compared to phosphate, vitamin D and parathyroid hormone. There was also positive correlation between vitamin D when compared with parathyroid hormone concentration. There was negative correlation between MDA and serum phosphate levels. Positive correlation was also observed between MDA and catalase, superoxide dismutase and gluthathione. However, a negative correlation was observed between serum glutathione and MDA concentration.

6.2 Conclusion

Serum levels of calcium, vitamin D, parathyroid hormone, calcitonin, malondialdehyde and activities of antioxidant enzymes were significantly influenced by trimesters of pregnancy and lactation in women in Zaria, but did not influence serum concentration of phosphate. Local base – line reference values for calcium, phosphate, vitamin D, parathyroid hormone and calcitonin for pregnant women were recorded. A very low prevalence of calcium, phosphate and vitamin D deficiency was recorded; although serum concentrations of calcium decreased across the trimesters and the period of lactation. Predictive equations for serum calcium levels during the different trimesters of pregnancy and the period of lactation were formulated. Age, parity, occupation, ethnic group and body mass index did not influence serum calcium and phosphate concentrations.

6.3 Recommendations

i. Due to the relative hypercalcaemia observed in this study, physicians and dieticians should be cautious in recommending calcium rich foods/supplements to pregnant women in Zaria and environs during antenatal visits.

ii. Determination of serum calcium and phosphate levels at different trimesters of pregnancy may be useful in deciding on the level of calcium supplementation, if any at all is required.

iii. Similar studies should be conducted in other geopolitical zones in order to determine calcium and phosphate levels in pregnant and lactating women and whether supplementation is necessary in specific areas or not.

iv. This is a hospital based study, it is recommended that a community based study should be carried out on a larger scale.

6.4 Contributions to Knowledge

i. The study established that serum concentration of calcium was significantly lower during the course of lactation than during the 3rd trimester pregnancy as it fell from 2.24 ± 0.14 mmol/L to 2.21 ± 0.17 mmol/L ($p = 0.01$, $f = 3.44$).

ii. Regression equations were estabilished for estimating calcium levels in pregnant and lactating women:

1st Trimester: Calcium = 3.036 – (0.317*Phosphate) – (0.00966 * Vit D) F (7.84); P = 0.002

2nd Trimester: Calcium = 2.784 - (0.253 * Phosphate) – (0.00395 * Vit D) F (10.78); P < 0.001

3rd Trimester: Calcium = 2.766 - (0.228 * Phosphate) – (0.00640 * Vit D) F (7.993); P < 0.001)

Lactating women: Calcium = 2.554 - (0.154 * Phosphate) – (0.00384 * Vit D) F(4.39); P = 0.028

iii. The study determined the relationship between oxidative stress and serum calcium biomarkers. Vitamin D correlated positively with superoxide dismutase ($r = 0.175$, $p = 0.05$) and glutathione peroxidase ($r = 0.227$, $p = 0.01$). Malondialdehyde also showed an inverse relationship with serum phosphate levels, ($r = - 0.189$, $p < 0.05$).

REFERENCES

Aebi, H., Wyss, S., Scherz, B., & Skvaril, F. (1974). Heterogeneity of erythrocyte catalase isolation and characterization of normal and variant erythrocyte catalase and their subunits. *European Journal of Biochemistry, 48(1),* 137-145.

Abrams, S. A. (2010). Calcium absorption in infants and small children: methods of determination and recent findings. *Nutrients 2(4),* 474–480,

Abrams, S. A. (2007). In utero physiology: role in nutrient delivery and fetal development for calcium, phosphorus, and vitamin D. *The American Journal of Clinical Nutrition 85(2),* 604S-607S.

Agbon, C. A., Akinyemi, C. O., Onabanjo, O. O. & Okeke, E. C. (2010). Nutrient composition and phytate-zinc molar ratio of prepared foods consumed by rural preschool children. *Nigerian Journal of Nutritional Sciences, 31*(*1*),15-18.

Ainy, E., Ghazi, A. A. M. & Azizi, F. (2006). Changes in calcium, 25(OH) vitamin D_3 and other biochemical factors during pregnancy. *Journal of Endocrinological Investigation, 29(4),* 303 – 307

Allgrove, J., Adami, S., Manning, R. M. & O'Riordan, J. L. (1985). Cytochemical bioassay of parathyroid hormone in maternal and cord blood. *Archives of Disease in Childhood, 60(2),* 110–115

Aloia, J. F., Dhaliwal, R., Shieh, A., Mikhail, M., Fazzari, M., Ragolia, L. & Abrams, S. A. (2014). Vitamin D supplementation increases calcium absorption without a threshold effect. *American Journal of Clinical Nutrition, 99(3),* 624–631

Alshahrani, F. & Aljohani, N. (2013). Vitamin D: deficiency, sufficiency and toxicity. *Nutrients, 5(9),* 3605-3616

Anai, T., Tomiyasu, T., Arima, K. & Miyakawa, I. (1999). Pregnancy-associated osteoporosis with elevated levels of circulating parathyroid hormone-related protein: a report of two cases. *The Journal of Obstetrics and Gynaecology, 25(1),* 63–67.

Asemi, Z., Hashemi, T., Karamali, M., Samimi, M. & Esmaillzadeh, A. (2014). Effects of vitamin D supplementation on glucose metabolism, lipid concentrations, inflammation and oxidative stress in gestational diabetes: a double-blind randomized controlled clinical trial. *American Journal of Clinical Nutrition, 98(6),* 1425–1432

Asemi, Z., Raygan, F., Bahmani, F., Rezavandi, Z., Talari, H. R., Rafiee, M., … Esmaillzadeh, A. (2016). The effects of vitamin D, K and calcium co-supplementation on carotid intima-media thickness and metabolic status in overweight type 2 diabetic patients with CHD. *116(2),* 286-93

Ardawi, M. S. & Nasrat, H. A. (1997). Calcium-regulating hormones and parathyroid hormone-related peptide in normal human pregnancy and postpartum: a longitudinal study. *European Journal of Endocrinology, 137(4),* 402–409

Atawodi, S. E. (2011). *Nigerian foodstuffs with prostate cancer chemopreventive polyphenols*. Paper presented at the conference of The Science of Global Prostate Cancer Disparities in Black Men, Jacksonville, FL. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3194188/pdf/1750-9378-6-S2-S9.pdf

Atkins, G. J. & Findlay, D. M. (2012) Osteocyte regulation of bone mineral: a little give and take. *Osteoporosis International*, 23(*8)*, 2067–2079.

Aurousseau, B., Gruffat, D. & Durand, D. (2006). Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant, *Reproduction Nutrition Development, 46(6),* 601-620

Jensen, R. G. (Ed.). (1995) *Major minerals and ionic constituents of human and bovine milk*. In: Handbook of Milk Composition. New York: Academy Press. pp. 593–619.

Avidime, O. M., Avidime, S., Olorunshola, K. V. & Dikko, A. A. U. (2011) Anogenital distance and umbilical cord testosterone level in newborns in Zaria, Northern Nigeria. *Nigerian Journal of Physiological Sciences, 26(1),* 023 – 028

Avidime, O. M. Olorunshola, K. V. & Dikko, A. A. U. (2011). Anogenital Distance of Newborns and Causes of Variation in Zaria, Northern Nigeria. *Asian Journal of Medical Sciences*, *3*(*2*), 74 – 78

Baran, D. T. & Braverman, L. E. (1991). Thyroid hormones and bone mass. *Journal of Clinical Endocrinology and Metabolism, 72(6),* 1182–1183

Barrett, K. E., Barman, S. M., Boitano, S. & Brooks, H. L. (Eds.). (2010). *Ganong's Review of Medical Physiology*. (23rd Ed.). McGraw Hill Companies, Inc.

Benali, A. I. & Demmouche, A. (2014). Calcium deficiency among pregnant women and their newborns in Sidi Bel Abbes region, Algeria. *Journal of Nutrition and Food Sciences*, *4*(*6*), 318.

Biberoglu, E., Biberoglu, K., Kirbas, A, Daglar, K., Genc, M., Avci, A. & Nuri Danisman, N. (2016). Circulating and myometrial markers of oxidative stress in pregnant women with fetal growth restriction. *42(1),* 29-35

Bikle, D. D., Gee, E., Halloran, B., Haddad & J. G. (1984) Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *The Journal of Clinical Investigation, 74(6),* 1966–1971,

Black, A. J., Topping, J., Durham, B., Farquharson, R. G. & Fraser, W. D. (2000). A detailed assessment of alterations in bone turnover, calcium homeostasis, and bone density in normal pregnancy. *Journal of Bone and Mineral Research, 15(3),* 557–563

Boass, A., Garner, S. C., Schultz., V. L. & Toverud, S. U. (1997) Regulation of serum calcitriol by serum ionized calcium in rats during pregnancy and lactation. *Journal of Bone and Mineral Research, 12(6),* 909–914

Bodnar, L. M., Simhan, H. N., Powers, R. W., Frank, M. P., Cooperstein, E. & Roberts, J. M. (2007). High prevalence of vitamin d insufficiency in black and white pregnant women residing in the northern united states and their neonates. *Journal of Nutrition, 137(2)*, 447-52

Body, J. J., Chanoine, J. P., Dumon, J. C. & F. Delange. (1993). Circulating calcitonin levels in healthy children and subjects with congenital hypothyroidism from birth to adolescence. *Journal of Endocrinology and Metabolism, 77(2)*, 565–567

Bolland, M. J., Grey, A., Avenell, A., Gamble, G. D., & Reid, I. R. (2011). Calcium supplements with or without Vitamin D and risk of cardiovascular events: reanalysis of the women's health initiative limited access dataset and meta-analysis. *British Medical Journal, 19(342)*, d2040. Doi: 10.1136/bmj.d2040.

Bolzetta, F., Veronese, N., De Rui, M., Berton, L., Carraro, S., Pizzato, S., … Sergi, G. (2014). Duration of breastfeeding as a risk factor for vertebral fractures. *Bone, 68*, 41–45. D oi: 10.1016/j.bone.2014.08.001

Bonacker, J., Janousek, M. & Krober, M. (2014). Pregnancy-associated osteoporosis with eight fractures in the vertebral column treated with kyphoplasty and bracing: a case report. *Archives of Orthopaedic and Trauma Surgery, 134(2)*, 173–179

Borkenhagen, J. F., Connor, E. L. & Stafstrom, C. E. (2013). Neonatal hypocalcemic seizures due to excessive maternal calcium ingestion. *Pediatric Neurology 48(6)*, 469–471

Boundless Biology (2017). 21st April 2017 accessed via https://www.boundless.com/biology/textbooks/boundless-biology-textbook/the-endocrine-system-37/regulation-of-body-processes-212/hormonal-control-of-blood-calcium-levels-800-12036/ @ 11.20hrs

Briana, D. D., Boutsikou, M., Baka, S., Hassiakos, D., Gourgiotis, D., Marmarinos, A., … Malamitsi-Puchner, A. (2007). N-terminal parathyroid hormone-related protein levels in human intrauterine growth restricted pregnancies. *Acta Obstetricia et Gynecologica Scandinavica, 86(8)*, 945–949

Buppasiri, P., Lumbiganon, P., Thinkhamrop, J.; Ngamjarus, C. & Laopaiboon, M. (2011). Calcium supplementation (other than for preventing or treating hypertension) for improving pregnancy and infant outcomes (review). *Cochrane Database System Rev*iew *2*(*10*): CD007079.

Cabiddu, G., Castellino, S., Gernone, G., Santoro, D., Giacchino, F., Credendino, O., … Piccoli, G. B. (2015). Best practices on pregnancy on dialysis: the Italian Study Group on Kidney and Pregnancy. *Journal of Nephrology, 28(3)*, 279–288

Campos-Obando, N., Oei, L., Hoefsloot, L. H., Kiewiet, R. M., Klaver, C. C., Simon, M. E. & Zillikens, M. C. (2014). Osteoporotic vertebral fractures during pregnancy: be aware of a potential underlying genetic cause. *Journal of Clinical Endocrinology and Metabolism, 99(4)*, 1107–1111.

Caprio, M., Mammi, C. & Rosano, G. M. (2012). Vitamin D: a novel player in endothelial function and dysfunction. *Archives of Medical Science, 8*(*1*), 4–5.

Chabas, J. F., Alluin, O., Rao, G., Garcia, S., Lavaut, M. N., Risso, J. J., … Feron, F. J. (2008). Vitamin D2 potentiates axon regeneration. *Neurotrauma, 25(10),* 1247-1256. doi: 10.1089/neu.2008.0593.

Chakravarti, B., Chattopadhyay, N. & Brown, E. M. (2012). Signaling through the extracellular calcium-sensing receptor (CaSR). *Advances in Experimental Medicine and Biology, 740,* 103 – 142

Chalon, S. & Garel, J. M. (1985). Plasma calcium control in the rat fetus. I. Influence of maternal hormones. *Biology of the Neonate, 48(6),* 313–322

Chen, S., Law, C. S., Grigsby, C. L., Olsen, K., Hong, T. T., Zhang, Y., … Gardner, D. G. (2011) Cardiomyocyte-specific deletion of the vitamin D receptor gene results in cardiac hypertrophy. *Circulation, 124*(*17*),1838-1847

Cho, G. J., Hong, S. C., Oh, M. J. & Kim, H. J. (2013). Vitamin D deficiency in gestational diabetes mellitus and the role of the placenta. *American Journal of Obstetrics and Gynecology, 209*(*6*), 560

Choe, E. Y., Song, J. E., Park, K. H., Seok, H., Lee, E. J., Lim, S. K. & Rhee, Y. (2012). Effect of teriparatide on pregnancy and lactation-associated osteoporosis with multiple vertebral fractures. *Journal of Bone and Mineral Metabolism 30(5)*: 596–601.

Cooper, C., Harvey, N. C, Javaid, M. K., Bishop, N. J., Kennedy, S., Papageorghiou, A. T., … Reid, D. M. (2016) Effectiveness of maternal vitamin D supplementation: a multicentre randomized, doubleblind, placebo controlled trial (MAVIDOS). *Lancet Diabetes & Endocrinology 4*(*5*), 393 – 402

Copp, D., Cameron, E. C., Cheney, B. A., Davidson, A. G. & Henze, K. G. (1962) Evidence for calcitonin — a new hormone from the parathyroid gland that lowers blood calcium. *Endocrinology, 70(5),* 638–649

Costante, G., Meringolo, D., Durante, C., Bianchi, D., Nocera, M., Tumino, S., … Filetti, S. (2007). Predictive value of serum calcitonin levels for preoperative diagnosis of medullary thyroid carcinoma in a cohort of 5817 consecutive patients with thyroid nodules. *Journal of Endocrinology and Metabolism, 92(2),* 450–455

Christakos, S. (2012). Mechanism of action of 1,25-dihydroxyvitamin D_3 on intestinal calcium absorption. *Reviews in Endocrine & Metabolic Disorders, 13(1),* 39–44.

Cross, N. A., Hillman, L.S., Allen, S. H., Krause, G. F. & Vieira, N. E.. (1995). Calcium homeostasis and bone metabolism during pregnancy, lactation and postweaning: a longitudinal study. *American Journal of Clinical Nutrition, 61(3),* 514–523

Cruikshank, D. P., Pitkin, R. M., Reynolds, W. A., Williams, G. A. & Hargis, G. K. (1980). Altered maternal calcium homeostasis in diabetic pregnancy. *Journal of Endocrinology and Metabolism, 50(2),* 264–267

Dardenne, O., Prud'homme, J., Hacking, S. A., Glorieux, F. H. & St-Arnaud, R. (2003). Correction of the abnormal mineral ion homeostasis with a high-calcium, high-phosphorus, high lactose diet rescues the PDDR phenotype of mice deficient for the 25-hydroxyvitamin D-1alpha-hydroxylase (CYP27B1). *Bone, 32(4),* 332–340

Dawodu, A. & Wagner, C. L. (2007) Mother-child vitamin d deficiency: an international perspective. *Archives of Disease in Childhood, 92,*737-40

Dawodu, A., Saadi, H. F., Bekdache, G., Javed, Y., Altaye, M. & Hollis, B. W. (2013). Randomized controlled trial (RCT) of vitamin D supplementation in pregnancy in a population with endemic vitamin D deficiency. *The Journal of Clinical Endocrinology and Metabolism, 98,* 2337–2346

Davey, R. A. & Findlay, D. M. (2013) Calcitonin: Physiology or Fantasy? *Journal of Bone and Mineral Research, 28*(*5*), 973 – 979. doi: 10.1002/jbmr.1869

Davey, R. A., Turner, A. G., McManus, J. F., Chiu, W. S., Tjahyono, F., Moore, A. J., … Zajac, J. D. (2008) The calcitonin receptor plays a physiological role to protect against hypercalcemia in mice. *Journal of Bone and Mineral Research, 23*(*8*), 1182–1193

Davis, O. K., Hawkins, D. S., Rubin, L. P., Posillico, J. T., Brown, E. M. & Schiff, I. (1988). Serum parathyroid hormone (PTH) in pregnant women determined by an immunoradiometric assay for intact PTH. *The Journal of Clinical Endocrinology and Metabolism, 67(4),* 850–852

Deftos, L. J., Weisman, M. H., Williams, G. W., Karpf, D. B, Frumar, A. M., Davidson, B. J., … Judd, H. L. (1980) Influence of age and sex on plasma calcitonin in human beings. *New England Journal of Medicine, 302(24),* 1351–1353

Del Rio, D., Stewart, A. J. & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism & Cardiovascular Diseases, 15(4),* 316-328

Demirel, N., Aydin, M., Zenciroglu, A., Okumus, N., Cetinkaya, S., Yildiz, Y. T., … Ipek, M. S. (2009). Hyperparathyroidism secondary to maternal hypoparathyroidism and vitamin D deficiency: an uncommon cause of neonatal respiratory distress. *Annals of Tropical Paediatrics, 29(2),* 149–154

Diaz de Barboza, G., Guizzardi, S. & Tolosa de Talamoni, N. (2015). Molecular aspects of intestinal calcium absorption. *World Journal of Gastroenterology, 21(23),* 7142–7154

Dubreuil, P., Arsenault, J. & Belanger, D. (2005) Biochemical reference ranges for groups of ewes of different ages. *Veterinary Record, 156(20),* 636–638

Dusso, A. S.; Brown, A. J. & Slatopolsky, E. (2005). Vitamin D. *American Journal of Physiology - Renal Physiology, 289(1),* 8 – 28

Dytfeld, J. & Horst-Sikorska, W. Pregnancy associated osteoporosis–a case report. (2012). *Ginekologia Polska, 83(05),* 377–379

Ekici F, Ozyurt B. & Erdogan, H. (2009). The combination of Vitamin D_3 and dehydroascorbic acid administration attenuates brain damage in focal ischemia. *Neurological Sciences, 30(3),* 207 – 212

El-Hajj Fuleihan, G. & Arnold A. (2013). *Pathogenesis and etiology of primary hyperparathyroidism.* Reteieved from http://ultra-medica.net/Uptodate21.6/contents/UTD.htm?29/41/30361/abstract/6

Eller-Vainicher, C., Ossola, M. W., Beck-Peccoz, P. & Chiodini, I. (2012). PTHrP-associated hypercalcemia of pregnancy resolved after delivery: a case report. *European Journal of Endocrinology, 166(4),* 753–756

Ellman, M. (1959). A spectrophotometric method for determination of reduced glutathione in tissues. *Analytical Biochemistry, 74,* 214-226.

Farrugia, W., Ho, P. W., Rice, G. E., Moseley, J. M., Permezel, M. & Wlodek, M. E. (2000). Parathyroid hormone-related protein (1–34) in gestational fluids and release from human gestational tissues. *Journal of Endocrinology 165,* 657–662

Faulkner, W. R. & King, J. W. (1970). *Manual of Clinical Laboratory Procedures*., Cleveland: Chemical Rubber Company. Pp 354.

Fedirko, V., Bostick, R. M., Long, Q., Flanders, W. D., McCullough, M. L., Sidelnikov, … Shaukat, A. (2010) Effects of supplemental Vitamin D and calcium on oxidative DNA damage marker in normal colorectal mucosa: A randomized clinical trial. *Cancer Epidemiology, Biomarkers & Prevention, 19(1),* 280-291.

Felsenfeld, A. J. & Levine, B. S. (2015) Calcitonin, the forgotten hormone: does it deserve to be forgotten? *Clinical Kidney Journal, 8(2),* 180–187

Food and Nutrition Board, Institute of Medicine (2004). *Dietary reference intakes (DRIs): Recommended intakes for individuals.* Washington, DC: National Academies of Sciences

Forman, J. P., Williams, J. S. & Fisher, N. D. (2010). Plasma 25-hydroxyvitamin D and regulation of the renin-angiotensin system in humans. *Hypertension 55*(*5*), 1283–1288.

Fridovich, I. (1989). Superoxide dismutases. An adaptation to a paramagnetic gas. *The Journal of Biological Chemistry*, *264*(*14*), 7761-7764

Fudge, N. J. & Kovacs, C. S. (2010). Pregnancy up-regulates intestinal calcium absorption and skeletal mineralization independently of the vitamin D receptor. *Endocrinology 151(3),* 886–895

Fukumoto, S. & Shimizu, Y. (2011) Fibroblast growth factor 23 as a phosphotropic hormone and beyond. *Journal of Bone and Mineral Metabolism 29*(*5*), 507-14

Fukumoto, S. (2014) Phosphate metabolism and vitamin D. *Bonekey Reports*. Retrieved from http://www.nature.com/bonekeyreports/2014/140205/bonekey2013231/full/bonekey2013231.html

Gallagher, J. C., Yalamanchili, V. & Smith, L. M. (2012). The effect of vitamin D on calcium absorption in older women. *Journal of Endocrinology and Metabolism 97(10),* 3550–3556

Gbadegesin, A., Sobande, A., Adedeji, O., Disu, E., Korede, O., Dosunmu, A. & Shakunle, A. (2017). Maternal serum vitamin D levels and pregnancy outcomes: from Lagos. *The Journal of Obstetrics and Gynaecology*, *37*(*1*), 25-28.

Gillette, M. E., Insogna, K. L., Lewis, A. M. & Baran, D. T. (1982) Influence of pregnancy on immunoreactive parathyroid hormone levels. *Calcified Tissue International 34(1),* 9–12

Gillies, B., Tonkin, B. A., Ma, Y., Kirby, B. J., St-Arnaud, R., Sims, N. A. & Kovacs, C. S. (2015). Pregnancy and post-lactation recovery rescue low bone mass and hypocalcemia in Cyp27b1 null mice that cannot make calcitriol (Abstract). *Journal of Bone and Mineral Research 30*

Gitto, E., Reiter, R. J., Karbownik, M., Tan, D. X., Gitto, P., Barberi, S. & Barberi I. (2002). Causes of oxidative stress in the pre and perinatal period. *Biology of the Neonate*, *81(3),* 146–157

Glerean, M., Furci, A., Galich, A. M., Fama, B. & Plantalech, L. (2010). Bone and mineral metabolism in primiparous women and its relationship with breastfeeding: a longitudinal study. *Medicina, 70(3),* 227–232

Glew, R. H., Crossey, M. J., Polanams, J., Okolie, H. I. & Vanderjagt, D. J. (2010) Vitamin D status of seminomadic Fulani men and women. *Journal of the National Medical Association, 102(6),* 485-90

Glorieux, F. H., Arabian, A. & Delvin, E. E. (1995). Pseudo-vitamin D deficiency: absence of 25-hydroxyvitaminD1 alpha-hydroxylase activity in human placenta decidual cells. *The Journal of clinical endocrinology and metabolism, 80(7)*: 2255–2258

Gomori, G. (1942) Modification of colorimetric phosphorus determination for use with photoelectric colorimeter. *Journal of Laboratory and Clinical Medicine*, *27,* 955-960.

Gooi, J. H., Pompolo, S., Karsdal, M. A., Kulkarni, N. H., Kalajzic, I., McAhren, S. H., … Sims, N. A. (2010). Calcitonin impairs the anabolic effect of PTH in young rats and stimulates expression of sclerostin by osteocytes. *Bone*, *46*(*6*),1486–97.

Gray, T. K., Lowe, W. & Lester, G. E. (1981). Vitamin D and pregnancy: the maternal-fetal metabolism of vitamin D. *Endocrine Reviews, 2(3),* 264–274

Guyton, A. C. & Hall, J. E. (2011). *Guyton and Hall Textbook of Medical Physiology*. Hall, J. E. (Ed.). Philadelphia: Saunders. 12th Ed pp 371 – 372

Hacker-Thompson, A., Schoeloetter, M. & Sellmeyer, D. (2012). Validation of a dietary vitamin D questionnaire using multiple diet records and the block 98 health habits and history questionnaire in healthy postmenopausal women in Northern California. *Journal of the Academy of Nutrition and Dietetics, 112* (*3*) 419 – 423

Hajsadeghi, S., Hejrati, M., Moghadami, S., Rismantab, S. & Namiranian, P. (2012). Dilated cardiomyopathy in two patients with xeroderma pigmentosum disease: A case report. *Acta Medica Iranica, 50*(*2*), 147-150

Halicker, H. D., Zhao, H., Li, J., Traganos, F., Studzinski, G. P. & Darzynkiewicz, Z. (2012). Attenuation of constitutive DNA damage signaling by 1,25-dihydroxyvitamin D_3. *Aging, 4(4),* 270-278

Hamilton, K., Tein, M., Glazier, J., Mawer, E. B., Berry, J. L., Balment, R. J., … Sibley, C. P. (2000). Altered calbindin mRNA expression and calcium regulating hormones in rat diabetic pregnancy. *Journal of Endocrinology, 164*, 67–76

Hawk, P. B.; Oser, B. L. & Summerson, W. H. (1951). Practical physiological chemistry, 12th ed. pp. 589, 839, 896. London: Churchill.

Heersche, J. N. M. (1992). Calcitonin effects on osteoclastic resorption: the 'escape phenomenon' revisited. *Journal of Bone and Mineral Research,16(3),* 174–177

Heidari, B., Heidari, P., Nourooddini, H. G. & Hajian-Tilaki, K. O. (2013). Relationship between parity and bone mass in postmenopausal women according to number of parities and age. *Journal of Reproductive Medicine, 58(9-10),* 389–394.

Heidari, B., Hosseini, R., Javadian, Y., Bijani, A., Sateri, M. H & Nouroddini, H. G. (2015). Factors affecting bone mineral density in postmenopausal women. *Archives of Osteoporosis, 10,* 15. doi: 10.1007/s11657-015-0217-4

Hicks, P. D., Hawthorne, K. M., Berseth, C. L., Marunycz, J. D., Heubi, J. E. & Abrams, S. A. (2012). Total calcium absorption is similar from infant formulas with and without prebiotics and exceeds that in human milk-fed infants. *BMC Pediatrics 12,* 118. Doi: 10.1186/1471-2431-12-118

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., … Weaver, C. M. (2012). Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited *The Journal of Clinical Endocrinology & Metabolism, 97*(*4*),1153-8

Holick, M. F., Biancuzzo, R. M., Chen, T. C., Klein, E. K., Young, A., Bibuld, D., … Tannenbaum, A. D. (2008). Vitamin D2 is as effective as vitamin D_3 in maintaining circulating concentrations of 25-hydroxyvitamin D. *Journal of Clinical Endocrinology and Metabolism, 93*(*3*), 677-81

Hollis, B. W. & Wagner, C. L. (2013). The role of the parent compound vitamin d with respect to metabolism and function: why clinical dose intervals can affect clinical outcomes. *Journal of Clinical Endocrinology & Metabolism, 98*(*12*), 4619–4628.

Hollis, B. W., Johnson, D., Hulsey, T. C., Ebeling, M. & Wagner, C. L. (2011) Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. *Journal of Bone and Mineral Research, 26(10)*, 2341–2357

Hoff, A. O., Catala-Lehnen, P., Thomas, P. M., Priemel, M., Rueger, J. M, Nasonkin, I., Gagel, R. F. (2002). Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. *Journal of Clinical Investigation 110*(*12*), 1849–1857.

Holt, E. (2013) Calcium physiology in pregnancy *Upto Date* retrieved from http://cursoenarm.net/UPTODATE/contents/mobipreview.htm?29/41/30367?source=see_link

Hosking, D. J. & Gilson, D. (1984) Comparison of the renal and skeletal actions of calcitonin in the treatment of severe hypercalcaemia of malignancy. *Quarterly Journal of Medicine, 53* (*211*):359–68.

Hunt, C. D. & Johnson, L. K. (2007) Calcium requirements: new estimations for men and women by cross-sectional statistical analyses of calcium balance data from metabolic studies. *The American Journal of Clinical Nutrition 86(4)*: 1054–1063.

Idonije, O. B., Festus, O., Okhai, O. & Akpamu, U. (2011). A comparative study of the status of oxidative stress in pregnant nigerian women. *Research Journal of Obstetrics and Gynecology*, *4*(*1*), 28-36.

Ikepkeazu, J. E., Ibegbu, M. D. & Ugwoke, H. A. (2010) Evaluation of serum calcium and inorganic phosphate levels in pregnant and lactating women in Enugu Metropolis. *Bioresearch 8* (*1*) 622 – 625.

Institute of Medicine. (2011) *Dietary Reference Intakes for Calcium and Vitamin D*. Washington, DC: The National Academies Press

Iwamoto, J., Sato, Y., Uzawa, M. & Matsumoto, H. (2012) Five-year follow-up of a woman with pregnancy and lactation-associated osteoporosis and vertebral fractures. *Journal of Therapeutics and Clinical Risk Management 8*, 195–199

Jimoh, A., Tanko, Y., Ahmed, A., Mohammed, A. & Ayo, J. O. (2015). Protective effect of resveratrol coadministration with high fat diet on blood glucose homeostasis and thyroid function in rabbits. *Cell Biology*, *3*(*1*), 19-24

Jurutka, P. W., Whitfield, G. K., Hsieh, J. C., Thompson, P. D., Haussler, C. A. & Haussler, M. R. (2001). Molecular nature of the vitamin D receptor and its role in regulation of gene expression. *Reviews in Endocrinology and Metabolic Disorders, 2*(*2*): 203–16

Kalra, S., Baruah, M. P., Sahay, R. & Kanishka, S. (2013). The history of parathyroid endocrinology *Indian Journal of Endocrinology and Metabolism, 17*(*2*), 320–322.

Kametas N, McAuliffe F & Krampl E (2003), Maternal electrolyte and liver function changes during pregnancy at high altitude. *Clinica Chimica Acta, 328(1-2),* 21-29

Kantham, L., Quinn, S. J., Egbuna, O. I., Baxi, K., Butters, R., Pang, J. L … Brown, E. M. (2009). The calcium-sensing receptor (CaSR) defends against hypercalcaemia independently of its regulation of parathyroid hormone secretion. *American Journal of Physiology - Endocrinology and Metabolism, 297(4),* E915–E923

Kawashima, H., Torikai, S. & Kurokawa, K. (1981) Calcitonin selectively stimulates 25-hydroxyvitamin D_3-1 alpha- hydroxylase in proximal straight tubule of rat kidney. *Nature, 291(5813),* 327–329

Kauppi, M., Heliovaara, M., Impivaara, O., Knekt, P. & Jula, A. (2011). Parity and risk of hip fracture in postmenopausal women. *Osteoporosis International 22(6),* 1765–1771

Kaur, M., Godber, I. M., Lawson, N., Baker, P. N., Pearson, D. & Hosking, D. J. (2003) Changes in serum markers of bone turnover during normal pregnancy. *Annals of Clinical Biochemistry, 40(5),* 508–513,

Kim, M. K., Yun, K. J., Kim, M. H., Lim, D. J., Kwon, H. S., Song, K. H., … Baek, K. H. (2015) The effects of thyrotropin-suppressing therapy on bone metabolism in patients with well-differentiated thyroid carcinoma. *Bone 71,* 101–105. DOI: http://dx.doi.org/10.1016/j.bone.2014.10.009

Kim, S., An, B. S., Yang, H. & Jeung, E. B. (2013) Effects of octylphenol and bisphenol A on the expression of calcium transport genes in the mouse duodenum and kidney during pregnancy. *Toxicology. 303,* 99-106

Khoo, C. C., Woo, J., Leung, P. C., Kwok, A. & Kwok, T. (2011). Determinants of bone mineral density in older postmenopausal Chinese women. *Climacteric 14(3),* 378–383

Khovidhunkit, W. & Epstein, S (1996). Osteoporosis in pregnancy. *Osteoporosis International, 6(5),* 345-354

Kirkwood, J. A. C. & Stern, B. R. (2003). *Essential Medical Statistics*. Massachussets: Blackwell publishing 2nd Ed. pp 9, 35

Kirby, B. J., Ma, Y., Martin, H. M, Buckle Favaro, K. L., Karaplis, A. C. & Kovacs, C. S. (2013) Upregulation of calcitriol during pregnancy and skeletal recovery after lactation do not require parathyroid hormone. *Journal of Bone and Mineral Research, 28(9),* 1987–2000

Kirby, B. J., Ardeshirpour, L., Woodrow, J. P., Wysolmerski, J. J., Sims, N. A., Karaplis, A. C. & Kovacs, C. S. (2011) Skeletal recovery after weaning does not require PTHrP. *Journal of Bone and Mineral Research 26(6),* 1242–1251

Knapen, M. F. C. M., Zusterzeel, P. L. M., Peters, W. H. M. & Steegers, E. A. P. (1999). Glutathione and glutathione-related enzymes in reproduction: A review. *European Journal Obstetrics, Gynaecology and Reproductive Biology, 82(2),* 171-184.

Ko, J. A., Lee, B. H., Lee, J. S. & Park, H. (2008). Effect of UV-B exposure on the concentration of vitamin D2 in sliced shiitake mushroom (lentinusedodes) and white button mushroom (*Agaricusbisporus*). *Journal Agriculture and Food Chemistry, 56(10),* 3671-3674.

Kolnick, L., Harris, B. D., Choma, D. P. & Choma, N. N. (2011). Hypercalcemia in pregnancy: a case of milk-alkali syndrome *Journal of General Internal Medicine 26(8),* 939–942

Kovacs, C. S. (2016) Maternal mineral and bone metabolism during pregnancy, lactation, and post-weaning recovery *Physiological Reviews, 96(2),* 449–547

Kovacs, C. S & Ralston, S. H. (2015). Presentation and management of osteoporosis presenting in association with pregnancy or lactation. *Osteoporosis Internationatal, 26*(*9*), 2223-2241. doi: 10.1007/s00198-015-3149-3.

Kovacs, C. S. (2014) Bone development and mineral homeostasis in the fetus and neonate: roles of the calciotropic and phosphotropic hormones. *Physiological Reviews, 94(4),* 1143–1218

Kovacs, C. S. (2011). Bone development in the fetus and neonate: role of the calciotropic hormones. *Current Osteoporosis Reports*, *9*(*4*), 274 - 83

Kovacs, C. S. (2008). Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies. *The American Journal of Clinical Nutrition, 88*(*2*), 5205 – 5285.

Kovacs, C. S. & Kronenberg, H. M. (1997). Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. *Endocrine Reviews 18(6),* 832–872

Kubota, M., Ohno, J., Shiina, Y. & Suda, T. (1982). Vitamin D metabolism in pregnant rabbits: differences between the maternal and fetal response to administration of large amounts of vitamin D_3. *Endocrinology, 110(6),* 1950–1956

Kudo, T., Miyauchi, A., Ito, Y., Yabuta, T., Inoue, H., Higashiyama, T., … Hirokawa, M. (2011). Amino N. Serum calcitonin levels with calcium loading tests before and after total thyroidectomy in patients with thyroid diseases other than medullary thyroid carcinoma. *Endocrine Journal 58(3),* 217–221

Kumar, A., Agarwal, K., Devi, S. G., Gupta, R. K. & Batra, S. (2010). Hypocalcemia in pregnant women. *Biological Trace Element Research, 136(1),* 26 – 32

Kumar, R., Abboud, C. F. & Riggs, B. L. (1980) The effect of elevated prolactin levels on plasma 1,25-dihydroxyvitamin D and intestinal absorption of calcium. *Mayo Clinic Proceedings, 55(1),* 51–53.

Larsson, S., Jones, H. A., Göransson, O., Degerman, E. & Holm, C. (2016). Parathyroid hormone induces adipocyte lipolysis via PKA-mediated phosphorylation of hormone-sensitive lipase. *Cellular Signalling, 28(3),* 204–213

Lee, M. Y., Park, J. H., Bae, K. S., Jee, Y. G., Ko, A. N., Han, Y. J., … Kang, S. J. (2014) Bone mineral density and bone turnover markers in patients on long-term suppressive levothyroxine therapy for differentiated thyroid cancer. *Annals of Surgical Treatment and Research, 86(2),* 55–60

Lee, J. M., Smith. J. R., Philip, B. L., Chen, T. C., Mathieu, J. & Holick, M. F. (2007). Vitamin D deficiency in a healthy group of mothers and newborn infants. *Clinical Pediatrics*, *46(1),* 42-44

Lee, S. H., Hong, M. K., Park, S. W., Park, H. M., Kim, J. & Ahn, J. (2013). A case of teriparatide on pregnancy-induced osteoporosis. *Journal of Bone Metabolism 20(2),* 111–114

Leitch, I. & Aitken, F. C. (1959). The estimation of calcium requirement: a re-examination. *Nutrition Abstracts and Reviews. Serie A, Human and Experimental, 29(2),* 393–411

Levine, B. S., Rodriguez, M. & Felsenfeld, A. J. (2014) Serum calcium and bone: Effect of PTH, phosphate, vitamin D and uremia. *Nefrologia*, *34*(*5*), 658 – 69.

Lindsay, K. L., Gibney, E. R., McNulty, B. A. & McAuliffe, F. M. (2014). Pregnant immigrant Nigerian women: an exploration of dietary intakes. *Public Health*, *128*(*7*), 647-53

Lindsay, K. L., E. R. Gibney & F. M. McAuliffe (2012). Maternal nutrition among women from Sub-Saharan Africa, with a focus on Nigeria, and potential implications for pregnancy outcomes among immigrant populations in developed countries. *Journal of Human Nutrition and Dietetics, 25(6),* 534–546

Liu, S., Zhu, W., Li, S., Ma, J., Zhang, H., Zhonghe, L., … Wei, S. (2016). Bovine parathyroid hormone enhances osteoclast bone resorption by modulating V-ATPase through PTH1R. *International Journal of Molecular Medicine, 37(2),* 284-292

Lombardi, G., Lanteri, P., Colombini, A. & Banfi, G. (2012). Blood biochemical markers of bone turnover: pre-analytical and technical aspects of sample collection and handling. *Clinical Chemistry and Laboratory Medicine 50(5),* 771–789

Lu, Z., Chen, T. C., Zhang, A., Persons, K. S., Kohn, N., Berkowitz, R., ... & Holick, M. F. (2007). An evaluation of the vitamin D_3 Content in fish: is the vitamin D content adequate to satisfy the dietary requirement for vitamin D? *Journal of Steroid Biochemistry Molecular Biology, 103 (3-5),* 642 –644

Lubetzky, R., Weisman, Y., Dollberg, S., Herman, L. & Mandel, D. (2010) Parathyroid hormone related protein in preterm human milk. *Breastfeeding Medicine, 5(2),* 67–69

Lundqvist, A., Sandström, H., Stenlund, H., Johansson, I. & Hultdin, J. (2016). Vitamin D status during pregnancy: A longitudinal study in swedish women from early pregnancy to seven months postpartum *Plos One, 11*(*3*), e0150385. Doi:10.1371/journal.pone.0150385

Lycans, D., Salloum, E., Wingate, M. K., Melvin, T., Buchanan, G. S. & Shuler, F. D. (2016). Vitamin D deficiency: "at risk" patient populations and potential drug interactions. *Marshall Journal of Medicine, 2(1),* 11 DOI: http://dx.doi.org/10.18590/mjm.2016.vol2.iss1.11

Madar, A.A., Stene, L.C. & Meyer, H. E. (2009). Vitamin D status among immigrant mothers from Pakistan, Turkey and Somalia and their infants attending child health clinics in Norway. *British Journal of Nutrition, 101*, 1052–1058. Doi:10.1017/S0007114508055712

Madukosiri C.H; Adoga G.I, (2011). Elemental status of pregnant and lactating women in Bassa LGA of Plateau state, Nigeria. *Pakistan Journal of Nutrition, 10*(*5*), 401 – 404.

Marini, F., Bartoccini, E., Cascianelli, G., Voccoli, V,. Baviglia, M. G., Magni, M. V., … Albi E. (2010). Effect of 1alpha,25-dihydroxyvitamin D_3 in embryonic hippocampal cells. *Hippocampus, 20*(*6*), 696-705. doi: 10.1002/hipo.20670.

Marthins, M. I. (2006). *Zaria and itsregions*. Department of Geography, Ahmadu Bello University Printing Press, Zaria. Pp 41

Marwaha, R. K., Tandon, N., Chopra, S., Agarwal, N., Garg, M.K., Sharma, B., … Puri, S. (2011). Vitamin D status in pregnant Indian women across trimesters and different seasons and its correlation with neonatal serum 25-hydroxyvitamin D levels. *British Journal of Nutrition, 106(9),* 1383–1389

Mizwicki, M. T., Liu, G., Fiala, M., Magpantay, L., Sayre, J., Siani, A., … Teplow, D. B. (2013). 1α,25-dihydroxyvitamin D_3 and resolving D1 retune the balance between amyloid-β phagocytosis and inflammation in Alzheimer's disease patients. *Journal of Alzheimer's Disease, 34*(*1*), 155-70. doi: 10.3233/JAD-121735.

Møller, U. K., Streym, S., Mosekilde, L., Heickendorff, L., Flyvbjerg, A., Frystyk, J., … Rejnmark, L. (2013) Changes in calcitropic hormones, bone markers and insulin-like growth factor I (IGF-I) during pregnancy and postpartum: a controlled cohort study. *Osteoporosis International, 24(4),* 1307–1320

Mori, T., Ishii, S., Greendale, G. A., Cauley, J. A., Ruppert, K., Crandall, C. J. & Karlamangla, A. S. (2015). Parity, lactation, bone strength, and 16-year fracture risk in adult women: findings from the Study of Women's Health Across the Nation (SWAN). *Bone, 73,* 160–166. doi:10.1016/j.bone.2014.12.013

Morley, R., Carlin, J. B., Pasco, J. A. & Wark, J. D. (2006) Maternal 25-hydroxyvitamin D and parathyroid hormone concentrations and offspring birth size. *Journal of Clinical Endocrinology and Metabolism, 91(3),* 906–912

Mulligan, M. L., Felton, S. K., Riek, A. E. & Bernal-Mizrachi, C. (2010). Implications of vitamin D deficiency in pregnancy and lactation. *American Journal of Obstetrics & Gynecology, 202*(*5*), 492e1 – e499

Nair-Shalliker, V., Armstrong, B. K. & Fenech, M. (2012). Does Vitamin D protect against DNA damage? *Mutation Research, 733(1-2),* 50-57

Nakayama, S., Yasui, T., Suto, M., Sato, M., Kaji, T., Uemura, H., … Irahara, M. (2011). Differences in bone metabolism between singleton pregnancy and twin pregnancy. *Bone, 49(3),* 513–519

National Bureau of Statistics (2015). 25th February 2015 accessed via http://www.nigerianstat.gov.ng/ @ 11.53hrs.

Naveh-Many, T., Silver, J. (1988) Regulation of calcitonin gene transcription by vitamin D metabolites in vivo in the rat. *Journal of Clinical Investigation, 81(1),* 270–273

Naveh-Many T, Raue F, Grauer A et al. (1992) Regulation of calcitonin gene expression by hypocalcemia, hypercalcemia, and vitamin D in the rat. *Journal of Bone and Mineral Research, 7(10),* 1233–1237

Nesby-O'Dell, S., Scanlon, K. S., Cogswell, M. E., Gillespie, C., Hollis, B. W., Looker, A. C., … Bowman, B. A. (2002). Hypovitaminosis D prevalence and determinants among African American and white women of reproductive age: third National Health and Nutrition Examination Survey, 1988-1994. *American Journal of Nutrition, 76*(*1*), 187-192

Nguyen, T. P. H., Yong, H. E. J., Chollangi, T., A. J. Borg, A. J., Brennecke, S. P. & Murthi, P. (2015). Placental vitamin D receptor expression is decreased in human idiopathic fetal growth restriction. *Journal of Molecular Medicine, 93*(*7*), 795 – 805

Norenstedt, S., Pernow, Y., Brismar, K., Saaf, M., Ekip, A., Granath, F., … Nilsson, I. L. (2013). Primary hyperparathyroidism and metabolic risk factors, impact of parathyroidectomy and vitamin D supplementation, and results of a randomized double-blind study. *European Journal of Endocrinology, 169(6),* 795–804

Ogamba, J. O., Ughachukwu, P. O. & Ezema, E. C. (2011). Effect of Cholecalciferol Overdosage on Pregnancy Outcome in White Albino Mice. *Annals of Medical and Health Sciences Research, 1*(*2*), 181–186

Ogbodo, S. O., Okaka, A. N. C., Nwagha, U. I. & Ejezie, F. E. (2014). Free radicals and antioxidants status in pregnancy: need for pre- and early pregnancy assessment. *American Journal of Medicine and Medical Sciences, 4*(*6*), 230-235

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(*2*), 351-358

Ojofeitimi, E. O., Ogunjuyigbe, P. O., Sanusi, R. A., Orji, E. O., Akinlo, A., Liasu, S. A. & Owolabi, O. O. (2008). Poor dietary intake of energy and retinol among pregnant women: implications for pregnancy outcome in Southwest Nigeria. *Pakistan Journal of Nutrition 7(3),* 480–484.

Okonofua, F., Houlder, S., Bell, J., & Dandona, P. (1986). Vitamin D nutrition in pregnant Nigerian women at term and their newborn infants. *Journal of Clinical Pathology, 39(6),* 650-653

On, J. S., Chow, B. K. & Lee, L. T. (2015). Evolution of parathyroid hormone receptor family and their ligands in vertebrate. *Frontiers in Endocrinology (Lausanne), 6,* 28. doi: 10.3389/fendo.2015.00028

O'Seaghdha, C. M., Yang, Q., Glazer, N. L., Leak, T. S., Dehghan, A., Smith, A. V., Köttgen, A. (2010). Common variants in the calcium-sensing receptor gene are associated with total serum calcium levels. *Human Molecular Genetics, 19*(*21*), 4296 – 303

Ozturk, C., Atamaz, F. C., Akkurt, H. & Akkoc, Y. (2014). Pregnancy-associated osteoporosis presenting severe vertebral fractures. *Journal of Obstetrics and Gynaecology Research, 40(1),* 288–292

Papadopoulou, A., Gole, E., Moutafi, A., Sfikas, C., Oehrl, W., Samiotaki M., … Nicolaidou, P. (2014). A Calcium sensing receptor in pregnancies complicated by gestational diabetes mellitus A. *Placenta, 35(8),* 632 – 638

Patil, S. B., Kodliwadmath, M. V. & Kodliwadmath, S. M. (2007). Study of oxidative stress and enzymatic antioxidants in normal pregnancy. *Indian Journal of Clinical Biochemistry, 22* (*1*) 135-137

Paton, L. M., Alexander, J. L., Nowson, C. A., Margerison, C., Frame, M. G., Kaymakci, B. & Wark, J. D. (2003). Pregnancy and lactation have no long-term deleterious effect on measures of bone mineral in healthy women: a twin study. *The American Journal of Clinical Nutrition, 77(3),* 707–714

Pearse, A. G. (1966). The cytochemistry of the thyroid C cells and their relationship to calcitonin. *Proceedings of the Royal Society B: Biological Sciences, 164(996),* 478–487

Pettifor, J. M. (2008). Vitamin D and/or calcium deficiency rickets in infants and children; a global perspective. *Indian Journal of Medical Research, 127(3),* 245-249

Phillips, A. J., Ostlere, S. J. & Smith, R. (2000). Pregnancy-associated osteoporosis: does the skeleton recover? *Osteoporosis International, 11(5),* 449–454

Potts, J. T. (1992). Chemistry of the calcitonins. *Journal of Bone and Mineral Research, 16*(*3*), 169–173

Potts, J. T Jr., Bringhurst, F. R., Gardella, T., Nussbaum, S., Segre, G. & Kronenberg, H. M. (1995). Parathyroid hormone: physiology, chemistry, biosynthesis, secretion, metabolism, and mode of action. In: *Endocrinology*, edited by DeGroot LJ. Philadelphia: Saunders, p. 920–966

Powe, C. E., Seely, E. W., Rana, S., Bhan, I., Ecker, J., Karumanchi, S. A. & Thadhani, R. (2010). First trimester vitamin D, vitamin D binding protein, and subsequent preeclampsia. *Hypertension, 56(4),* 758–763

Quan-Sheng, D. & Miller S. C. (1989). Calciotrophic hormone levels and calcium absorption during pregnancy in rats. *American Journal of Physiology - Endocrinology and Metabolism, 257(1),* E118–E123

Qing, H., Ardeshirpour, L., Pajevic, P. D., Dusevich, V., Jahn, K., Kato, S., … Bonewald, L. F. (2012). Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. *Journal of Bone and Mineral Research*. *27*(*5*), 1018–29.

Rajagopalan, H., & Lengauer, C. (2004). Aneuploidy and cancer. *Nature*, *432*(*7015*), 338-341.

Rci.rutgers.edu accessed on 11th August, 2017 at http://www.rci.rutgers.edu/~uzwiak/AnatPhys/Endocrine_System.html

Reddy, G. S., Norman, A. W, Willis, D. M., Goltzman, D., Guyda, H., Solomon, S., … Mayer, E. (1983). Regulation of vitamin D metabolism in normal human pregnancy. *The Journal of Clinical Endocrinology & Metabolism, 56(2),* 363–370.

Ritchie, L. D., Fung, E. B., Halloran, B. P., Turnlund, J. R., Van Loan, M. D., Cann, C. E. & King, J. C. (1998). A longitudinal study of calcium homeostasis during human pregnancy and lactation and after resumption of menses. *American Journal of Clinical Nutrition, 67(4),* 693–701

Rodriguez, M. Felsenfeld, A. J., Torres, A. T. Pederson, L. & Llach, F. (1991). Calcitonin, an important factor in the calcaemic response to parathyroid hormone in the rat. *Kidney International, 40(2),* 219–225

Ross, A. C., Manson, J.E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., … Shapses, S. A. (2011). The report on dietary reference intakes for calcium and vitamin D from the institute of medicine: what clinicians need to know. *The Journal of Clinical Endocrinology & Metabolism, 96*(*1*), 53 – 58

Ross, A. C., Taylor, C. L.. Yaktine, A. L. & Del Valle, H. B. (Eds.) (2011). Dietary Reference Intakes for Calcium and Vitamin D. *Institute of Medicine*. Washington, D. C. : National Academies Press Pp 383 – 393

Roland-Zejly, L., Moisan, V., St-Pierre, I. & Bilodeau, J. F. (2011). Altered placental glutathione peroxidase mRNA expression in preeclampsia according to the presence or absence of labor. *Placenta*, *32*(*2*), 161-167

Roth, D. E., Al Mahmud, A., Raqib, R., Akhtar, E., Perumal, N., Pezzack, B. & Baqui, A. H. (2013). Randomized placebo-controlled trial of high-dose prenatal third-trimester vitamin D_3 supplementation in Bangladesh: the AViDD trial. *Nutrition Journal, 12,* 47. doi: 10.1186/1475-2891-12-47.

Sachan, A., Gupta, R., Das, V., Agarwal, A., Awasthi, P. K. & Bhatia, V. (2005). High prevalence of vitamin D deficiency among pregnant women and their newborns in Northern India. *The American Journal of Clinical Nutrition, 81(5),* 1060 – 1064

Sanchez, P. A., Idrisa, A., Bobzom, D. N., Airede, A., Hollis, B. W., Liston, D. E., … Glew, R. H. (1997). Calcium and vitamin D status of pregnant teenagers in Maiduguri, Nigeria. *Journal of the National Medical Association, 89(12),* 805 – 811

Sato, K. (2008). Hypercalcemia during pregnancy, puerperium, and lactation: review and a case report of hypercalcemic crisis after delivery due to excessive production of PTH-related protein (PTHrP) without malignancy (humoral hypercalcemia of pregnancy). *Endocrine Journal 55(6)*: 959–966.

Semins, M. J. & Matlaga, B. R. (2014). Kidney stones during pregnancy. *Nature Reviews Urology, 11(3),* 163–168

Schnatz, P. F., Marakovits, K. A. & O'Sullivan, D. M. (2010). Assessment of postmenopausal women and significant risk factors for osteoporosis. *Obstetrical & Gynecological Survey, 65(9),* 591–596

Schoenmakers, I., Jarjou, L.M., Goldberg, G. R., Tsoi, K., Harnpanich, D. & Prentice, A. (2013). Acute response to oral calcium loading in pregnant and lactating women with a low calcium intake: a pilot study. *Osteoporosis International, 24(8),* 2301–2308

Schogl, M. & Holick, M. F. (2014). Vitamin D and neurocognitive function *Journal of Clinical Interventions in Aging*. (*2014*)*9,* 559–568

Scott, Walsh. (1994). Lipid peroxidation in pregnancy. *Hypertension in Pregnancy 13*(*1*), 1-32

Shand, A. W., Nassar, N., Von Dadelszen, P., Innis, S. M. & Green, T. J. (2010). Maternal vitamin D status in pregnancy and adverse pregnancy outcomes in a group at high risk for pre-eclampsia. *British Journal of Obstetrics and Gynaecology, 117(13),* 1593–1598

Shagina, N. B., Tolstykh, E. I., Fell, T. P., Harrison, J. D., Phipps, A. W. & Degteva, M. O. (2007). In utero and postnatal haemopoietic tissue doses resulting from maternal ingestion of strontium isotopes from the Techa River. *Radiation Protection Dosimetry, 127(1-4),* 497–501

Shao, H., Tao, M., Fan, Y., Jing, J. & Lu, J. (2012). Vitamin D levels and other factors related to bone mineral density during pregnancy. *Australian and New Zealand Journal of Obstetrics and Gynaecology, 52(6),* 571–575

Seely, E. W., Brown, E. M., DeMaggio, D. M., Weldon, D. K. & Graves, S. W. (1997). A prospective study of calciotropic hormones in pregnancy and post partum: reciprocal changes in serum intact parathyroid hormone and 1,25-dihydroxyvitamin D. *American Journal of Obstetrics & Gynecology, 176(1 Pt. 1),* 214–217

Shibata, M., Suzuki, A., Sekiya, T., Sekiguchi, S., Asano, S., Udagawa, Y., Itoh, M. (2011). High prevalence of hypovitaminosis D in pregnant Japanese women with threatened premature delivery. *Journal of Bone and Mineral Research, 29(5),* 615–620

Shilbayeh, S. (2003). Prevalence of osteoporosis and its reproductive risk factors among Jordanian women: a cross-sectional study. *Osteoporosis International, 14(11),* 929–940

Silva, O. L., Titus-Dillon, P, Becker, K. L., Richard, H. Snider, and Charles F Moore (1981) Increased Serum Calcitonin in Pregnancy *Journal of the National Medical Association, 73*(*7*), 649–652

Sofronescu, A. G. (2015) Staros, E. B. (Ed.) *Medscape* retrieved from emedicine.medscape.com/article/2087580-overview

Strid, H., Bucht, E., Jansson, T., Wennergren, M. & Powell, T. L. (2003). ATP dependent Ca2þ transport across basal membrane of human syncytiotrophoblast in pregnancies complicated by intrauterine growth restriction or diabetes. *Placenta, 24*(*5*), 445 – 452

Suhail, M., Patil, S., Khan, S. & Siddiqui, S. (2010). Antioxidant Vitamins and Lipoperoxidation in Non-pregnant, Pregnant, and Gestational Diabetic Women: Erythrocytes Osmotic Fragility Profiles. *Journal of Clinical Medicine Research 2*(*6*), 266–273

Sweeney, L. L., Malabanan, A. O. & Rosen, H. (2010). Decreased calcitriol requirement during pregnancy and lactation with a window of increased requirement immediately post partum. *Endocrine Practice 16*(*3*), 459–462

Sypniewska, G., Pollak, J., Strozecki, P., Camil, F., Kretowicz, M., Janikowski, G., … Manitius, J. (2014). 25-Hydroxyvitamin D, biomarkers of endothelial dysfunction and subclinical organ damage in adults with hypertension. *American Journal of Hypertension*. *27(1)* doi:10.1093/ajh/hpt174

Talmage, R. V., Grubb, S. A., Norimatsu, H. & Vanderwiel, C. J. (1980). Evidence for an important physiological role for calcitonin. *Proceedings of the National Academy of Sciences, 77(1),* 609–613

Taylor, R. N. & Badell, M. L. (2011). *The endocrinology of pregnancy. In: Greenspan's Basic and Clinical Endocrinology*, Gardner, D. G., Shoback, D. G. (Eds.) .New York: Lange Medical Books/McGraw-Hill, (9th ed.). p. 553–572

Tiegs, R. D, Body, J. J., Barta, J. M & Heath, H. (1986). Secretion and metabolism of monomeric human calcitonin: effects of age, sex, and thyroid damage. *Journal of Bone and Mineral Research, 1*(*4*), 339–349

Tiwari, A. K. M., Mahdi, A. A., Zahra, F., Chandyan, S., Srivastava, V. K. & Negi, M. P. S. (2010). Evaluation of oxidative stress and antioxidant status in pregnant anaemic women. *Indian Journal of Clinical Biochemistry, 25(4),* 411-418

Tolstykh, E. I., Degteva, M. O., Vorobiova, M. I. & Kozheurov, V. P. (2001). Fetal dose assessment for the offspring of the Techa Riverside residents. *Radiation and Environmental Biophysics, 40*(*4*), 279–286

Torres, A., Rodriguez, M., Felsenfeld, A., Martin-Malo, A. & Llach, F. (1991). Sigmoidal relationship between calcitonin and calcium: studies in normal, parathyroidectomized, and azotemic rats. *Kidney International, 40(4),* 700–704

Turner, A. G., Tjahyono, F., Chiu, W. S., Skinner, J., Sawyer, R., Moore, A. J., … Davey, R. A. (2011). The role of the calcitonin receptor in protecting against induced hypercalcemia is mediated via its actions in osteoclasts to inhibit bone resorption. *Bone*, *48*(*2*), 354–61.

Ugwa, E., Gwarzo, M. & Ashimi, A. (2014). Oxidative stress and antioxidant status of pregnant rural women in north-west Nigeria: prospective cohort study. *Journal of Maternal - Fetal Neonatal Medicine, 28(5),* 544 - 547 doi:10.3109/14767058.2014.924102

Van der meer, I.., Karamali, N. S., Boeke, A. J., Lips, P., Middelkoop, B. J., Verhoeven, I. & Wuister, J. D. (2006). High prevalence of vitamin D deficiency in pregnant non-western women in the Hague, Netherlands. *The American Journal of Clinical Nutrition, 84*(*2*), 350-353.

VanHouten, J. N. & Wysolmerski, J. J. (2003). Low estrogen and high parathyroid hormone-related peptide levels contribute to accelerated bone resorption and bone loss in lactating mice. *Endocrinology 144*(*12*), 5521–5529

Verhaeghe, J., Allewaert, K., Van Herck, E., Van Bree, R., Van Assche, F. A. & Bouillon, R. (1994). 1,25-Dihydroxyvitamin D_3 and osteocalcin in maternal and fetal guinea pigs. *Journal of Bone and Mineral Research, 26(3),* 261–273,

Verhaeghe, J. & Bouillon, R. Calciotropic hormones during reproduction. (1992) *Journal of Steroid Biochemistry and Molecular Biology, 41*(*3 – 8*), 469–477,

Wagner, C. L., Baggerly, C., McDonnell, S. L., Baggerly, L., Hamilton, S. A., Winkler, J., … Hollis, B. W. (2015). Post-hoc comparison of vitamin d status at three timepoints during pregnancy demonstrates lower risk of preterm birth with higher vitamin d closer to delivery. *The Journal of Steroid Biochemistry and Molecular Biology, 148,* 256–260. doi:10.1016/j.jsbmb.2014.11.013.

Wagner, C. L., McNeil, R., Hamilton, S. A., Winkler, J., Rodriguez, C., Warner, G., & Hollis, B. W. (2013). A randomized trial of vitamin D supplementation in 2 community health center networks in South Carolina. *American Journal of Obstetrics & Gynecology*, *208*(*2*),137, e1–e13

Wagner, C. L. & Greer, F. R. (2008). Prevention of ricketts and vitamin D deficiency in infants, children and adolescents. *Peadiatrics*, *112*(*5*), 1142-1152

Wang, J., Yang, F., Mao, M., Liu, D., Yang, H. & Yang, S. (2010). High prevalence of vitamin d and calcium deficiency among pregnant women and their newborns in Chengdu, China. *World Journal of Pediatrics*, *6*(*3*), 265-267

Wang, W., Pang, C. C. P., Rogers, M. S. & Chang, A. M. Z. (1996). Lipid peroxidation in cord blood at birth. *American Journal of Obstetrics & Gynaecology, 174(1),* 62–65

Wittelsberger, A. & Rosenblatt, M. (2008). *Parathyroid hormone-receptor interactions*. In: Principles of Bone Biology, Bilezikian, J. P., Raisz, L. G. & Martin, T. J. (Eds.) New York: Academic (3rd ed.). p. 595–637

Wolf, G (2004). The Discovery of Vitamin D: The Contribution of Adolf Windaus . *Journal of Nutrition, 134*(*6*), 1299–1302

Woodrow, J. P., Sharpe, C. J., Fudge, N. J., Hoff, A. O., Gagel, R. F. & Kovacs, C. S. (2006). Calcitonin plays a critical role in regulating skeletal mineral metabolism during lactation. *Endocrinology, 147(9),* 4010–4021

Yadav, B., Paneri, S. & Yadav, S. (2014). Evaluation of oxidative stress and urinary calcium creatinine ratio in pregnancy induced hypertension. *Global Journal of Medical Research (E) Gynecology and Obstetrics*, *2*(*1*), 2249-2461

Yaqub, L. S.; Mshelia, W. P. & Ayo, J. O. (2014). Erythrocyte Osmotic Fragility and Hematological Responses of Horses Administered Ascorbic Acid and Exposed to Road Transportation. *Journal of Equine Veterinary Science, 34*(*11*), 1324 - 1328

Yates, N., Crew, R. C., Wyrwoll, C. S. (2017) Vitamin D deficiency and impaired placental function: potential regulation by glucocorticoids? *Reproduction, 153*(*5*), R163 – R171

Zarattini, G., Buffoli, P., Isabelli, G. & Marchese, M. (2014). Pregnancy-associated osteoporosis with seven vertebral compression fractures, a case treated with strontium ranelate. *Clinical Cases in Mineral and Bone Metabolism, 11*(*2*), 139–141

Zhang, J. Y., Lucey, A. J., Horgan, R., Kenny, L. C. & Kiely, M. (2014). Impact of pregnancy on vitamin D status: a longitudinal study. *British Journal of Nutrition, 112*(*7*), 1081–1087

Zhang, C.; Williams, M. A.; Sanchez, S. E.; King I. B. & Ware-Jauregui S., Larrabure, G., … Leisenring, W. M. (2001). Plasma concentrations of carotenoids, retinol and tocopherols in pre-eclamptic and normotensive pregnant women. *American Journal of Epidemiology*, *153*(*6*), 572-580

Zhang, Y. Y., Liu, P. Y. & Deng, H. W. (2003). The impact of reproductive and menstrual history on bone mineral density in Chinese women. *Journal of Clinical Densitometry, 6*(*3*), 289–296

Zhang, Q. L., Zhou, X. J. & Hong, J. G. (2009). Effect of Vitamin D supplementation in early life on the expression of interleukin-10 and intercellular adhesion molecule-1 in rat asthma model. *Zhonghua Er Ke Za Zhi*, 47(10), 735-739.

Appendix I

Prevalence of calcium, phosphate, vitamin D, parathyroid hormone, and calcitonin deficiency among pregnant women in Zaria, Nigeria.

Parameter	Normal Range	Minimum concentration	Maximum concentration	Hypo (<)	Hyper (>)
Calcium mmol/L	2.1– 2.5	2.00	2.56	Hypocalcaemia (1.76%)	Hypercalcaemia (5.31%)
Phosphate mmol/L	0.83–1.43	0.78	1.34	Hypophosphataemia (1.76%)	Hyperphosphataemia (0%)
Vitamin D µg/L	30.27– 48.31	40.89	45.93	Hypovitaminosis D (6.19%)	Hypervitaminosis D (21.24%)
PTH pg/ml	23.93–57.65	17.73	67.69	Hypoparathyroidism (0.88%)	Hyperparathyroidism (7.97%)
Calcitonin ng/ml	1.65–5.29	1.47	8.25	Hypocalcitoninaemia (0%)	Hypercalcitoninaemia (1.76%)

Appendix II

Mean (±) serum concentration of MDA, superoxide dismutase, glutathione peroxidase and catalase in control, pregnant and lactating women in Zaria, Nigeria.

Parameters	MDA (nmol/mg protein)	SOD (U/ml)	GPx (U/ml)	CAT (U/ml)
Control (n = 15)	35.03±102.25	6.74±0.74	11.71±1.83	7.63±1.28
First trimester (n = 40)	39±89.32	7.13±1.07	12.44±3.3	5.16±0.70
Second trimester (n = 41)	49.81±278.84	9.82±0.90	15.57±2.77	17.56±1.81
Third trimester (n = 53)	42.36±221.24	8.29±0.37	12.01±4.03	10.04±0.93
Lactating group (n = 30)	76.44±188.98**	14.87±1.61***	47.43±2.00***	3.14±0.78

Key: ** = $p < 0.01$ when compared to control, *** = $p < 0.001$

Appendix III

Age distribution of mean (±SD) serum concentration of calcium (mmol/l) in control, pregnant and lactating women in Zaria, Nigeria.

	Age Range (years)				
Trimester	**16 – 20 yrs**	**21 – 25 yrs**	**26 – 30 yrs**	**31 – 35 yrs**	**36 – 40 yrs**
Control	-	2.4±0.00 (n = 4)	2.26±0.12 (n = 7)	2.37±0.4 (n = 4)	-
1st Trimester	2.24±0.12 (n = 5)	2.30±0.12 (n = 7)	2.35±0.12 (n = 8)	2.36±0.07 (n = 5)	2.30±0.04 (n = 4)
2nd Trimester	2.24±0.25 (n = 6)	2.30±0.17 (n = 13)	2.35±0.14 (n = 6)	2.33±0.17 (n = 9)	2.31±0.13 (n = 4)
3rd Trimester	2.18±0.17 (n = 6)	2.20±0.08 (n = 17)	2.28±0.14 (n = 14)	2.24±0.17 (n = 8)	2.38±0.32 (n = 6)
Lactating Group	2.27±0.06 (n = 5)	2.15±0.13 (n = 6)	2.24±0.16 (n = 6)	2.13±0.20 (n = 4)	2.33±0.24 (n = 5)

Appendix IV

Age distribution in serum concentration of phosphate (mmol/l) in control, pregnant and lactating women in Zaria, Nigeria.

	Age Range (years)				
Group	**16 – 20 yrs**	**21 – 25 yrs**	**26 – 30 yrs**	**31 – 35 yrs**	**36 – 40 yrs**
Control	-	1.24±0.1 (n = 4)	1.17±0.11 (n = 7)	1.07±0.20 (n = 4)	-
1st Trimester	1.07±0.14 (n = 5)	0.93±0.11 (n = 7)	1.03±0.15 (n = 8)	1.04±0.11 (n = 5)	1.00±0.14 (n = 4)
2nd Trimester	1.17±0.078 (n = 6)	1.09±0.08 (n = 17)	1.00±0.12 (n = 14)	1.06±0.13 (n = 8)	1.18±0.16 (n = 6)
3rd Trimester	1.21±0.09 (n = 5)	1.04±0.14 (n = 6)	1.05±0.14 (n = 6)	1.13±0.14 (n = 4)	1.05±0.27 (n = 5)
Lactating Gp	0.91±0.00 (n = 2)	1.09±0.13 (n = 6)	1.08±0.14 (n = 6)	1.20±0.12 (n = 4)	1.11±0.18 (n = 3)

Appendix V

Age distribution in serum concentration of vitamin D (µg/l) in control, pregnant and lactating women in Zaria, Nigeria

	Age Range (years)				
Group	**16 – 20 yrs**	**21 – 25 yrs**	**26 – 30 yrs**	**31 – 35 yrs**	**36 – 40 yrs**
Control		33.00±0.5 (n = 1)	40.71±4.07 (n = 7)	41.00±3.74 (n = 4)	
1st Trimester	44.20±6.57 (n = 5)	43.29±2.25 (n =7)	37.88±3.23 (n =8)	39.00±4.30 (n =5)	43.25±4.72 (n =4)
2nd Trimester	49.50±17.68 (n = 2)	50.54±27.03 (n = 13)	50.83±25.33 (n = 6)	40.56±13.15 (n = 9)	41.75±10.53 (n = 4)
3rd Trimester	45.33±6.532 (n = 6)	42.06±6.87 (n = 17)	43.57±9.46 (n = 14)	42.88±10.08 (n = 8)	31.00±12.73 (n = 2)
Lactating Group	47.00±0.05 (n = 2)	37.50±19.02 (n = 6)	38.83±3.43 (n = 6)	58.25±38.68 (n = 4)	41.67±15.04 (n = 3)

Appendix VI

Age distribution in serum concentration of parathyroid hormone (pg/ml) in control, pregnant and lactating women in Zaria, Nigeria.

	Age Range				
Group	**16 – 20 yrs**	**21 – 25 yrs**	**26 – 30 yrs**	**31 – 35 yrs**	**36 – 40 yrs**
Control		35.00±0.00 (n = 1)	42.57±10.25 (n = 7)	40.00±8.76 (n = 4)	
1st Trimester	37.60±2.70 (n = 5)	43.00±8.54 (n = 7)	40.50±6.82 (n = 8)	40.80±5.81 (n = 5)	40.50±8.54 (n = 4)
2nd Trimester	40.50±20.51 (n = 2)	45.00±22.46 (n = 13)	52.33±17.97 (n = 6)	44.78±14.19 (n = 9)	43.50±12.37 (n = 4)
3rd Trimester	43.50±6.69 (n = 6)	40.83±12.89 (n = 17)	43.00±7.18 (n = 14)	42.00±13.92 (n = 8)	35.00±5.66 (n = 2)
Lactating Gp	51.00±0.00 (n = 2)	44.17±9.45 (n = 6)	44.00±6.33 (n = 6)	49.25±24.51 (n = 4)	37.67±14.57 (n = 3)

Appendix VII

Age distribution of serum concentration of calcitonin (ng/L) in control, pregnant and lactating women in Zaria, Nigeria.

	Age Range (years)				
Group	**16 – 20 yrs**	**21 – 25 yrs**	**26 – 30 yrs**	**31 – 35 yrs**	**36 – 40 yrs**
Control		4.20±0.00	3.20±0.83	3.57±1.00	
1st Trimester	2.92±1.03 (n =5)	2.47±1.23 (n =7)	3.30±0.74 (n = 8)	3.46±0.63 (n = 5)	3.20±0.66 (n = 4)
2nd Trimester	4.70±0.28 (n = 2)	5.08±6.64 (n = 13)	2.62±0.48 (n = 6)	3.53±0.89 (n = 9)	3.28±0.60 (n = 4)
3rd Trimester	2.85±0.80 (n = 6)	3.25±0.90 (n = 17)	3.26±0.87 (n = 14)	2.73±0.87 (n = 8)	4.40±1.56 (n = 2)
Lactating Group	3.60±0.57 (n = 2)	3.03±0.57 (n = 6)	2.90±0.48 (n = 6)	3.30±1.22 (n = 4)	3.40±1.25 (n = 3)

Appendix VIII

Effect of parity on serum concentration of calcium, phosphate, vitamin D, parathyroid and calcitonin in control, pregnant and lactating women in Zaria, Nigeria.

Parity	Calcium (mmol/L)	Phosphate (mmol/L)	Vitamin D (µg/L)	PTH (pg/ml)	Calcitonin (ng/L)
Control (n = 6)	2.22±0.14	1.12±0.19	40.00±5.57	43.33±5.86	4.13±0.96
0 – 3 (n = 108)	2.27±0.14	1.08±0.14	42.39±12.47	42.15±8.30	3.20±0.82
4 – 6 (n = 27)	2.28±0.16	1.05±0.14	46.11±18.93	45.07±16.35	4.04±4.66
7 – 10 (n = 6)	2.32±0.20	1.08±0.21	38.17±5.57	42.00±8.30	3.57±1.33

Key: PTH - Parathyroid hormone

Appendix IX

Mean (±) serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin concentration among different ethnic groups of pregnant and lactating women in Zaria, Nigeria.

Ethnic Group	Calcium (mmol/L)	Phosphate (mmol/L)	Vitamin D (µg/L)	PTH (pg/ml)	Calcitonin (ng/L)
Hausa (n = 82)	2.27±0.14	1.07±0.15	43.48±12.92	42.95±11.74	3.50±2.74
Yoruba (n = 14)	2.29±0.12	1.07±0.12	44.86±18.84	44.64±15.89	3.03±0.72
Ibo (n = 12)	2.29±0.11	1.11±0.15	36.50±11.51	41.17±8.57	3.46±0.83
Others (n = 34)	2.29±0.17	1.08±0.14	43.03±14.71	41.83±12.75	3.19±1.01

Key: PTH - Parathyroid hormone

Appendix X

Mean (± SD) serum concentration on oxidative stress biomarkers among different ethnic groups of pregnant and lactating women in Zaria, Nigeria.

Ethnic Group	Catalase (u/mg)	SOD (U/ml)	GSH (ug/ml)	MDA (nmol/mg protein)
Hausa (n = 82)	8.97±6.35	9.56±3.70	18.31±16.78	498.05±407.05
Yoruba(n = 14)	34.37±97.99	8.50±3.71	17.96±20.37	542.18±387.62
Ibo (n = 12)	7.81±4.52	10.55±7.85	17.58±20.83	481.48±357.48
Others (n = 34)	6.31±3.67	8.52±7.21	14.95±11.86	370.27±127.83

Key: SOD = Superoxide dismutase, GSH = Glutathione, MDA = Malondialdehyde

Appendix XI

Mean (± SD) calcium, phosphate, vitamin D, parathyroid hormone and calcitonin concentration among different occupational groups of pregnant and lactating women in Zaria, Nigeria.

Occupation	Calcium (mmol)	Phosphate (mmol)	Vitamin D (µg/L)	PTH (pg/ml)	Calcitonin (ng/L)
Housewife (n=71)	2.27±0.15	1.07±0.14	45.00±15.79	42.59±13.77	3.53±2.95
Handwork (n=43)	2.29±0.12	1.07±0.14	41.51±13.60	43.77±10.94	3.16±0.86
Civil Servant (n=28)	2.27	1.09±0.15	39.79±7.29	41.32±9.23	3.32±0.82

Appendix XII

Mean (± SD) Serum concentration of oxidative stress biomarkers among different occupational groups of pregnant and lactating women in Zaria, Nigeria.

Occupation	Catalase (u/mg)	SOD (U/ml)	GSH (ug/ml)	MDA (nmol/mg protein)
Housewife (n=71)	14.77±43.31	9.31±3.78	17.16±16.60	514.19±456.80
Handwork (n=43)	6.63±4.42	8.93±4.95	17.77±16.55	429.05±224.55
Civil Servant (n=28)	7.09±4.65	10.08±7.50	18.30±17.07	448.71±238.04

Key:
SOD = Superoxide dismutase, GSH = Glutathione, MDA = Malondialdehyde

Appendix XIII

Mean (± SD) Serum concentration of calcium, phosphate, vitamin D, parathyroid hormone and calcitonin concentration in relation to body mass index category of pregnant and lactating women in Zaria, Nigeria.

BMI(Kg/m^2) Category	Calcium (mmol/L)	Phosphate (mmol/L)	Vitamin D (µg/L)	PTH (pg/ml)	Calcitonin (ng/L)
Normal (18.5-24.9) (n=50)	2.26±0.18	1.07±0.16	46.42±17.86	44.58±14.05	3.70±3.49
Obese (≥30.0) (n=20)	2.27±0.12	1.07±0.16	43.10±8.11	42.65±8.62	3.28±0.66
Overweight (25.0-29.9) (n = 36)	2.30±0.11	1.07±0.13	41.06±13.96	42.64±13.62	3.42±0.83
Underweight (<18.5) (n = 4)	2.23±0.14	1.05±0.13	46.50±6.56	39.50±13.18	3.08±0.63

Appendix XIV

Effect of Residential Location on Serum Concentration Calcium, Phosphate, Vitamin D, Parathyroid Hormone and Calcitoninin pregnant and lactating women in Zaria, Nigeria.

	Residential Location	
Parameter	**ABU**	**T/Wada**
Calcium (mmol/L)	2.29±0.14	2.27±0.15
Phosphate (mmol/L)	1.09±0.14	1.06±0.14
Vitamin D (µg/L)	43.31±16.97	42.55±10.46
PTH (pg/L)	43.74±13.22	41.75±11.03
Calcitonin (ng/L)	3.10±0.86	3.63±2.87

Appendix XV

AHMADU BELLO UNIVERSITY, ZARIA
DEPARTMENT OF HUMAN PHYSIOLOGY
FACULTY OF MEDICINE
PROFORMA FOR STUDY ON EVALUATION OF BIOMARKERS OF CALCIUM REGULATION AND OXIDATIVE STRESS IN PREGNANT WOMEN IN ZARIA, NIGERIA

A. PERSONAL DATA

Name: _____
Serial No._____ Name of Hospital_____
SUBJECT
Participant' s Age_____ Parity_____ Tribe_____
Occupation_____
Resident Location_____

B. ANTHROPOMETRIC DATA

Height ___ Weight __ B/Group__ Blood Pressure____Gestational Age (Weeks) __

Husband's Age____ Husband's Occupation_____ Husband's Tribe_____

C. ANTENATAL HISTORY

What medication(s) was received in the current pregnancy (list)
_____, _____, _____,
_____
Are you currently taking other medication? Yes ( ) No ( )
If yes, _____ (type of drug) for how long?_____
Any illness(es) 3 months prior to pregnancy? Yes ( ) No ( )
If yes, type of illness_____
Any illness in current pregnancy Yes ( ) No ( )
If yes please state illness_____
What medication was given _____

D. LABORATORY DATA

i. Serum ionized calcium concentration _____
ii. Serum phosphate concentration _____
iii. Serum vitamin D concentration _____
iv. Serum parathyroid hormone concentration _____
v. Serum calcitonin concentration _____
vi. Serum malondialdehyde concentration _____
vii. Serum catalase concentration _____
viii. Serum superoxide dismutase concentration _____
ix. Serum glutathione peroxidase concentration _____

THANK YOU

Appendix XXVI



Plate I: Student running samples through the audicom auto-electrolytes analyzer

Appendix XXVII

Ethical Clearance Certificate

HEALTH RESEARCH ETHICS COMMITTEE
AHMADU BELLO UNIVERSITY TEACHING HOSPITAL
SHIKA - ZARIA, NIGERIA.
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*Our Ref:*_____ *Date:*_____

Your Ref: ABUTH/HREC/CL/05 8th September, 2015

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Evaluation of Biomakers of Calcium Regulation and Oxidative Stress in Pregnant Women in Zaria, Nigeria.

ABUTH Ethics Committee assigned number:	-	**ABUTHZ/HREC/K30/2014**
Name of the principal Investigator:	-	**Mrs, Ohunene Makoju Avidime**
Address of the Principal Investigator:	-	**Dept. of Human Physiology, ABU Zaria,**
Date of receipt of valid application:	-	**15/8/ 2015**
Date of meeting when final determination On ethical approval was made:	-	**4th & 5th September, 2015**

This is to inform you that the research described in the submitted protocol, the consent forms and other participant information materials have been reviewed and ***given full approval by the Health Research Ethics Committee.***

Please note: this approval dates from **8th September, 2015 -8th September, 2016**

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: http://www.nhrec.net.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.