ASSESSMENT OF THE RELATIONSHIP BETWEEN SOME TRACE ELEMENTS AND ANTIOXIDANT ENZYMES IN UNDER-FIVE CHILDREN WITH PROTEIN-ENERGY MALNUTRITION

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF SCIENCE DEGREE IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
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NOVEMBER, 2014

DECLARATION

I hereby declare that this thesis entitled **"Assessment of the relationship between some trace elements and antioxidant enzymes in under-five children with protein-energy malnutrition"** is a record of my own research work under the supervision of Dr. K.M. Anigo, Dr. S.A. Akuyam and Prof. G.O. Ogunrinde.

The information obtained from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been presented elsewhere for the award of a degree in any other University.

Njoku, Stella Ujunwa

| --- | --- | --- |
| Name of student | Signature | Date |

CERTIFICATION

This thesis titled **"Assessment of the relationship between some trace elements and antioxidant enzymes in under-five children with protein-energy malnutrition"** meet the regulations governing the award of the degree of Masters in Science (Biochemistry) of Ahmadu Bello University, Zaria and is approved for its contributions to knowledge and literary presentations.

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DEDICATION

This research work is dedicated to Almighty God for His inspiration and kindness in my life and to every member of Kevin Njoku's family for their all round wonderful support in my life.

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ABSTRACT

Assessment of the relationship between some trace elements and antioxidant enzymes was carried out on 98 under-five children with protein-energy malnutrition (PEM) and 98 age- and sex-matched apparently healthy children (control). The malnourished children involve those with Marasmus, Kwashiorkor and Marasmic-kwashiorkor. Venous blood (2ml) was collected from both PEM children and control for biochemical analysis using standard methods. Results obtained show that mean serum total protein (55.76±3.95) and albumin (26.43±2.78) levels and superoxide dismutase (SOD) (1.87±0.32) and glutathione peroxidase (GPx) (42.38±5.03) activities in malnourished children were significantly lower ($p<0.05$) than in the control. Mean serum zinc (Zn) concentrations (8.37±4.25) in malnourished children were significantly higher ($p<0.05$) than in the control (5.14±2.39), but mean serum copper (Cu) concentrations in malnourished (2.40±1.12) children were lower than in the control (2.82±1.18). There were correlations between these serum levels of trace elements (Zn and Cu) and antioxidant enzymes (SOD and GPx) in children with PEM and control. Marasmus (SOD-Zn: 0.03, SOD-Cu: 0.16, GPx-Zn: -0.14, GPx-Cu: 0.05), kwashiorkor (SOD-Zn: -0.39, SOD-Cu: -0.39, GPx-Zn: -0.54, GPx-Cu: -0.31), marasmic-kwashiorkor (SOD-Zn: -0.31, SOD-Cu: -0.51, GPx-Zn: -0.41, GPx-Cu: -0.48) and control (SOD-Zn: 0.12, SOD-Cu: 0.07, GPx-Zn: -0.07, GPx-Cu: -0.08). This study points to the fact that children with PEM are predisposed to high oxidative stress due to an increase in free radical production and decrease in antioxidant defense system. Therefore, routine laboratory investigation of antioxidants should be done for effective management of PEM.

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ABBREVIATIONS

ABUTH	Ahmadu Bello University Teaching Hospital
ANOVA	Analysis of Variance
BCG	Bromocresolgreen
Cu	Copper
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
ICH	Institute of Child Health
n	Sample Size
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
NO	Nitric Oxide
NaOH	Sodium Hydroxide
$O_2^{\cdot -}$	Superoxide Radical
OH	Hydroxyl Radical
p	p- value
PEM	Protein-Energy Malnutrition
R	Coefficient of Correlation
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SPSS	Statistical Package for Social Sciences
Zn	Zinc

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Severe malnutrition is common among developing countries both in rural and urban areas **(**Psaki *et al.,* 2012)**.** It is responsible for at least half of the 7.6 million child's deaths each year in developing countries (Park *et al.,* 2012). Children who are poorly nourished suffer up to 160 days of illness each year **(**UNICEF, 2008). Malnutrition magnifies the effects of every disease, including measles and malaria. The estimated proportions of deaths in which malnutrition is an underlying cause are diarrhoea (61%), malaria (57%), pneumonia (52%) and measles (45%) (Black *et al.,* 2003). Protein-energy malnutrition (PEM) is one of the most prevalent and devastating forms of malnutrition in the world (Whitney and Rolfes, 2008).

PEM is defined by the WHO as the cellular imbalance between the supply of nutrients and energy and the body's demand for them to ensure growth, maintenance, and specific functions (WHO, 1993). It has long been recognized as a common problem, especially for children in the developing countries whose nutritional intake is deficient for socioeconomic reasons (Collins *et al.,* 2006). PEM results from inadequate intake and absorption which may be due to diseases, insufficient household food security, inadequate maternal and child care, poor sanitation and ignorance (UNICEF, 1990). The earliest symptoms include subtle changes in the mood of the child while further changes include loss of appetite and interest in the surroundings, which lead to decreased social interaction with peers or siblings (Allen, 1995). When PEM becomes more severe, it has adverse effects on the child's cognitive and behavioural development, both in the short and long term (Mendez and Linda, 1999). It may also affect children's mental performance by other indirect mechanisms such as social and economic disadvantages (Johnston and Low,

1987), differences in parental education (Levine and Levine, 1991), years of schooling (Ceci, 1991), inadequate attention or affection from caregivers and other environmental factors .

Marasmus, kwashiorkor and marasmic-kwashiorkor are clinical forms of severe PEM (Scrimshaw and Viteri, 2010). Marasmus is characterized by muscle wasting, anaemia, severe weight loss, growth impairment as well as dry and thin hair (Jahoor *et al.,* 2008). Kwashiorkor is characterized by apathy, increased susceptibility to infection, hypoalbuminemia, oedema, weight loss, growth impairment as well as dry and brittle hair and skin lesions (Jahoor *et al.,* 2008). However, the clinical onset of kwashiorkor usually takes place in a shorter period of time as compared to marasmus. Marasmic-kwashiorkor occurs when there are symptoms of both marasmus and kwashiorkor (Wellcome, 1970).

Of all children under the age of five who suffer from PEM in developing countries, about 38% are stunted (low height-for-age), 31% are underweight (low weight-for-age) and 9% are wasted (low weight-for-height) (Brabin and Coulter, 2003). In Nigeria, about 52.6% of under-five children are stunted, 35.1% are underweight and 19.9% are wasted (NDHS, 2008). In Kaduna state, 6.8% prevalence of PEM was reported in Zaria (Alegbejo and Yakubu, 1993).

Free radicals are chemicals or molecular fragments that have a charge due to an excess or deficient number of electrons (Riley, 1994). Examples of free radicals are the superoxide anions, hydroxyl radicals, transition metals such as nitric acid and ozone. Free radicals are highly unstable because they have one or more unpaired electron, to regain its stability, the free radicals quickly find a stable but vulnerable compound from which to steal or grab an electron (Martin *et al.,* 2003). With the loss of an electron, the formerly stable molecule becomes a free radical itself and steals an electron from another nearby molecule. Thus, an electron snatching chain reaction

occurs with free radicals producing more free radicals thereby damaging the cells, proteins and DNA (Martin *et al.,* 2003).

Malnutrition leads to depletion of hepatic antioxidant stores and enhances hepatic release of free radicals (Robinson *et al.,* 1996). The harmful effects of these free radicals have been documented in children with PEM which is responsible for cell damage leading to oedema, fatty liver and skin lesions (Golden *et al.,* 1990).

An antioxidant defense system exists in the cells to keep the concentration of these free radicals at a non-harmful level. Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) are directly involved in the detoxification of free radicals through catalytic action (Stahl and Sies, 1997). These antioxidant enzymes are synthesized by the body but the trace elements (Selenium, Zinc and Copper) needed as cofactors must be supplied by the diet. Selenium (Se) is a cofactor in glutathione peroxidase (Chan *et al.,* 1998). The superoxide dismutase present in the cytosol contains zinc (Zn) and copper (Cu) (Fridovich, 1986). Reduced levels of these antioxidant enzymes (SOD and GPx) and their cofactors have been found in children with severe PEM (Sive *et al.,* 1993; Houssaini *et al.,* 1997) which results in the buildup of free radicals. In addition to trace elements that serve as cofactors of antioxidative enzymes, the diets also provide antioxidant vitamins (vitamin A, C and E) that react directly with free radicals in a non catalytic manner (Halliwell, 1995).

1.2 Statement of the Research Problem

Malnutrition remains a major health burden in developing countries. It is recognized globally as the most important risk factor for illness and death, contributing to half of death in young children worldwide (W.H.O, 2002). PEM has a great adverse effect on health resulting in poor

growth, impaired immunologic factors, irritability, apathy and delayed cognitive development (Ugwuja *et al.*,2007). Oedema, skin lesion, fatty liver found in severe protein-energy malnourished children has been reported to be as a result of the harmful effects of free radicals (Golden *et al.,* 1990) and reduced level of antioxidant defense system. Therefore, there is need to carry out a comprehensive study that will provide information for the better management of malnourished children.

1.3 Justification for the Study

Although evidence on the relationship between trace elements and PEM is known (Ugwuja *et al.,* 2007), there is no sufficient evidence or data on the relationship between trace elements and antioxidant enzymes among under-five children with PEM in Nigeria. Therefore, the results of this study could broaden the understanding of the relationship between trace elements and antioxidant enzymes in protein-energy malnourished children in Nigeria with a view to formulating nutrition intervention programmes and policies targeted at reducing the prevalence of this disease and its detrimental effect among under-five children.

1.4 Aim and Objectives of the Study

The aim of the present study was to establish the relationship between some trace elements and antioxidant enzymes in under-five children with PEM.

The specific objectives of the present study were as follows:

1. To determine the demographic and feeding characteristics of patients with PEM and the socio-economic characteristics of the parents and controls.

2. To determine the concentration of serum total protein, albumin, trace elements (zinc and copper), and serum antioxidant enzymes (superoxide dismutase and glutathione peroxidase) levels in patients with PEM and controls.
3. To compare the results of serum total protein, albumin, trace elements (zinc and copper) and antioxidant enzymes (superoxide dismutase and glutathione peroxidase) obtained from patients with PEM and controls.
4. To establish the relationship between some trace elements (copper and zinc) and antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in patients with PEM and controls.

CHAPTER TWO

LITERATURE REVIEW

Severe malnutrition is one of the most important underlying causes of child mortality in developing countries, particularly during the first 5 years of life (UNICEF, 2008). The major causes for this are poverty, world conflicts, lack of education, natural disasters and poor access to health (Leonor *et al.,* 2011)

Undernutrition is a consequence of consuming little energy and other essential nutrients or using or excreting them more rapidly than they can be replaced (UNICEF, 2006). This state of malnutrition is often characterized by infections and diseases as it intensifies the effect of every disease (UNICEF, 2006). PEM is one of the most prevalent and devastating forms of malnutrition in the world (Whitney and Rolfes, 2008).

PEM is defined by the WHO as the cellular imbalance between the supply of nutrients and energy and the body's demand for them to ensure growth, maintenance, and specific functions (WHO, 1993). It can be caused, primarily, by an inadequate diet or, secondarily, by deficiency in gastrointestinal absorption and/or increase in demand, or even, by an excessive excretion of nutrients (Leonor *et al.,* 2011). PEM usually manifests early in children between 6 months and 2 years of age and is associated with early weaning, delayed introduction of complementary foods, a low-protein diet and severe or frequent infections (Kwena *et al.,* 2003). Nearly one-third of children in the developing world are malnourished (Ahmed *et al.,* 2009). The spectrum of PEM ranges in severity from mild, through moderate to severe degrees. The mild and most of the cases of moderate PEM are subclinical and can only be detected by anthropometric and biochemical

tests (El-Mougy, 1999). These early stages are characterized by growth failure and possibly some retardation of mental development (El-Mougy, 1999).

PEM appears in three principal clinical forms: marasmus, characterized by chronic wasting condition and a gross underweight status that is habitually associated with early weaning; kwashiorkor, characterized by moderate growth retardation, changes to hair and skin colour, oedema, moon face, and hepatosplenomegaly; and marasmic kwashiorkor, characterized by severe wasting and the presence of oedema. Marasmus appears by caloric and protein insufficiency, whereas kwashiorkor develops from protein deficiency (Waterlow, 1996).

PEM often coexist with micronutrient deficiencies (Bryan *et al.*, 2004). Malnutrition results from multiple deficiencies not necessarily limited to lack of proteins or calories but also involving other associated or separated factors such as selenium, copper and zinc (Ballabriga, 1985).

2.1 Prevalence of PEM

Malnutrition remains one of the most common causes of morbidity and mortality among children throughout the world (WHO, 2002). It is responsible for at least half of the 7.6 million child's deaths each year in developing countries (Park *et al.*, 2012).

Globally in 2011, an estimated 101 million children under 5 years of age were underweight, or approximately 16 per cent of children under 5. Underweight prevalence is highest in South Asia, which has a rate of 33 per cent, followed by sub-Saharan Africa, at 21 per cent. South Asia has 59 million underweight children, while sub-Saharan Africa has 30 million (UNICEF, 2013). In 2009, the WHO estimated that 27% of children in developing countries under the age of 5 years are malnourished. Approximately 178 million children (32% of children in the developing world) suffer from chronic malnutrition. Although the prevalence of childhood malnutrition is decreasing in Asia, countries in South Asia still have both the highest rates of malnutrition and

the largest numbers of malnourished children. Indeed, the prevalence of malnutrition in India, Bangladesh, Afghanistan and Pakistan (38–51%) is much higher than in sub-Saharan Africa (26%) (Ahmed *et al.*, 2009).

The prevalence of PEM is lowest during the first six months of life, due to the fact that breast feeding is the rule in this period and is considered satisfactory from the nutritional point of view (Osman *et al.,* 1978). On the other hand, in the second six mouths of life, the incidence of malnutrition increases as breast milk becomes insufficient to meet all dietary requirements, and supplements given are mostly watery fluids of negligible nutritional value (Osman *et al.,* 1978).

2.2 Assessment of Nutritional Status

Nutritional status assessment is the evaluation of an individual's health status from a nutrition perspective. It is a means by which the undernourished (or overnourished) child can be identified, the nutritional effect of therapy and the efficacy of nutritional interventions monitored and prevalence of under or overnutrition in a group established (Reilly, 1998).

There are four principal approaches to nutritional assessment: clinical, anthropometric, dietary and biochemical (Whitney and Rolfes, 2008).

2.2.1 Clinical assessment

Severe nutritional deprivation is easily detectable on clinical examination (Weaver, 1998). Physical findings like changes to hair and skin colour are most valuable in revealing problems for other assessment techniques, to confirm or in confirming other assessment measures such as anthropometric, dietary and biochemical nutritional Assessment (Weaver, 1998).

2.2.2 Anthropometric assessment

Anthropometric measurement such as body weight and height reveal the nutritional status of an individual **(**Pinstrup *et al.,* 1993**)**. Anthropometrically, PEM is defined as measurements that fall below 2 standard deviations under the normal weight-for-height **(**Pinstrup *et al.,* 1993**)**. Wasting indicates recent weight loss, whereas stunting usually results from being chronically undernourished. In clinical practice, these anthropometric indices (body weight and height) are useful but can be limited (Potter *et al.,* 1995). For example, when a child has fluid retention or large solid tumour, such condition can affect weight-based anthropometric indices. The mid-arm circumference and skin fold thickness has been proposed for the identification and classification of PEM of various degrees and severity (Potter *et al.,* 1995).

The advantages of these anthropometric measurements are that they are simple, inexpensive, portable and suitable.

2.2.3 Dietary intake assessment

Assessment of nutritional status based on dietary intake provides a record of an individual's eating habits and food intake and can help identify possible nutrient imbalances (Whitney and Rolfes, 2008). Recording dietary intake can be done using various ways such as 24 hours recall, the usual intake record and food frequency questionnaire (Whitney and Rolfes, 2008). Food models or photos and measuring devices can also help to identify the type of foods and quantities consumed (Whitney and Rolfes, 2008).

2.2.4 Biochemical assessment

Biochemical nutritional assessments are based on analysis of blood and urine samples, which contain nutrients, enzymes, and metabolites that reflect nutritional status. These analyses are especially useful in helping to detect subclinical malnutrition by uncovering early signs of malnutrition before the clinical signs of deficiency appear (Robinson, 1998).

2.3 Classification of PEM

In 1970, Wellcome classified PEM based on the presence or absence of oedema and the deficit of body weight.

Table 2.1: Wellcome's Classification of PEM

State of malnutrition	Body weight % of standard	Oedema
Simple underweight	80 – 60%	Absent
Marasmus	< 60%	Absent
Kwashiorkor	80 – 60%	Present
Marasmic-kwashiorkor	< 60%	Present

Adapted from Wellcome (1970)

PEM is frequently classified on the basis of deficits of weight-for-age (w/a) or height-for-age (Gomez *et al.*, 1956; Waterlow *et al.*, 1977). In this system, children are classified into three groups according to malnutrition severity based on their weight compared to the weight average for their age. First degree or mild cases of malnutrition include children whose weights are 75–90% of the average weight. Children with second degree or moderate cases have weights

between 60–74% of the average, and children with third degree or severe malnutrition weigh less than 60% (Gomez *et al.,* 1956).

Table 2.2: Gomez's Classification of PEM

% of expected weight for age	Grade of PEM
75% - 90%	First degree
60 % - 74%	Second degree
< 60%	Third degree

Gomez Classification of PEM (Gomez *et al,* 1956)

The major disadvantage of this classification is that it assumes all children of certain age should have the same weight. It also includes actively malnourished children and those who are underweight as a result of malnutrition of the past. However, Gomez classification overestimates the prevalence of malnutrition (Kiju *et al.*, 1991).

2.3.1 Marasmus

Marasmus is a clinical syndrome of severe PEM caused by severe deprivation of food over a long period of time (chronic PEM). It is characterized by extreme growth failure, wasted muscle and subcutaneous fat, with weight of 60% or less than that expected for age (Jelliffe and Jelliffe, 1991). Marasmus occurs most commonly in children from 6 to 18 months of age in all the over populated and impoverished areas of the world (Kwena *et al.*, 2003). This is because children in those areas simply do not have enough to eat and subsist on diluted cereal drinks that supply scanty energy and protein of low quality; such food can barely sustain life and much less support

growth (Kwena *et al.*, 2003). Consequently, marasmic children look like little old people (just skin and bone) (Whitney and Rolfes, 2008).

2.3.2 Kwashiorkor

Kwashiorkor is a clinical syndrome of severe PEM caused by recent deprivation of food (acute PEM). Kwashiorkor is a Ghanaian word that refers to the birth position of a child and is used to describe the illness a child develops when the next one is born (Krawinkel, 2003). When a woman who has been nursing her first child bears a second child, she weans the first child so as to breastfed the second child. The first child suddenly switched from nutrient-dense, protein-rich milk to a starchy, protein-poor cereal and soon begins to sicken and die (Krawinkel, 2003). Kwashiorkor occurs most commonly in children between 18 months to two years of age (Krawinkel, 2003).

Kwashiorkor usually develops rapidly as a result of protein deficiency or more commonly, is precipitated by an illness such as measles or other infection (Krawinkel, 2003). Other factors such as aflatoxins (a fungal toxin found on grains stored in warm moist condition), may also contribute to the development of, or symptoms that accompany kwashiorkor (Krawinkel, 2003).

A loss of body weight and fat is usually not as severe in kwashiorkor as in marasmus, but some muscle wasting may occur (Krawinkel, 2003). Protein and hormones that previously maintained fluid balance diminish, and fluid leaks into the interstitial spaces. The child's limbs and abdomen becomes swollen with oedema, a distinguishing feature of kwashiorkor (Krawinkel, 2003). A fatty liver develops due to lack of the protein carrier that transports fat out of the liver (Krawinkel, 2003). The fatty liver lacks enzymes to clear metabolic toxins from the body, so their harmful effects are prolonged (Krawinkel, 2003). Inflammation in response to these toxins

and infections further contributes to the oedema that accompanies kwashiorkor. Without sufficient tyrosine to make melanin, the child's hair loses its color, and inadequate protein synthesis leaves the skin patchy and scaly, often with sore that fail to heal. The lack of proteins to carry or store iron leaves iron free. Unbound iron is common in children with kwashiorkor and may contribute to their illness and death by promoting bacterial growth and free radical damage (Krawinkel, 2003).

2.3.3 Marasmic-kwashiorkor

Marasmic-kwashiorkor is characterized by both symptoms of marasmus and kwashiorkor such as oedema of kwashiorkor with the wasting of marasmus (Waterlow, 1996). Most often, the child suffers the effect of both malnutrition and infections (Whitney and Rolfes, 2008).

2.4 Complications of PEM

2.4.1 Infection

Infection is a major complication of PEM and may occur without the classical signs and symptoms (W.H.O, 2000). In PEM, antibodies that fight against invading bacteria are degraded to produce amino acids for other uses, leaving the malnourished child vulnerable to infections (Bloss *et al.,* 2004). Blood proteins, including haemoglobin are no longer synthesized, so the child becomes anaemic and weak (Bloss *et al.*, 2004). Infection can suppress appetite and directly affect nutrient metabolism, leading to poor nutrient utilization (Bloss *et al.,* 2004).

2.4.2 Diarrhoea and dehydration

Dysentery, an infection of the digestive tract, causes diarrhoea, further depleting the body nutrients and fluids leading to dehydration (Reid, 2002). In marasmic child once infection sets in, kwashiorkor often follows, and the immune response weakens further (Reid, 2002).

2.4.3 Heart failure

The combination of infections, fever, fluid imbalance and anaemia often leads to heart failure and occasionally sudden death (Wharton, 1991). Acute congestive heart failure occurs in children with PEM due to excess sodium intake. Moreover, heart failure in kwashiorkor, anaemia, vigorous refeeding and volume overload may also be involved (Wharton, 1991).

2.4.4 Hypothermia

Severe deprivation of food in children with PEM usually makes them to experience cold due to low energy level which is responsible for heat generation. Body temperature below 35.5^{o}C may occur due to impaired thermoregulatory mechanism and severe infections (Torun and Chew, 1994).

2.5 Prevention and Treatment of PEM

The treatment of PEM is to feed children a ready to-use therapeutic food (RUTP) until they have gained adequate weight. In some cases, it may be possible to construct an appropriate therapeutic diet using locally available nutrients-dense foods with added micronutrients supplements. However, this approach requires very careful monitoring because nutrient adequacy is hard to achieve (W.H.O, 2007). Prevention of PEM requires provision of a good supply of food and prompt treatment of gastroenteritis with oral rehydration (Weaver, 1994). Prolonged breast feeding up to two years should be encouraged along with the use of locally available protein foods. Continuation of breast feeding during diarrhoea episodes should also be encouraged (Victora *et al.*, 2000). Promotion of health education, immunization programs, family planning, and regular supervision of child's nutritional status are important tools in the early detection of moderate deficiency, allowing advice and treatment to be given well before occurance of severe

malnutrition (Wharton and Weaver, 1998; Ojofeitimi *et al.,* 2003). Infections are also common in severely malnourished children thus, antibiotics are often routinely recommended (WHO, 2000).

2.6 Free Radicals

Free radicals can be defined as reactive chemical species having a single unpaired electron in an outer orbit (Riley, 1994). This unstable configuration creates energy which is released through reactions with adjacent molecules, such as proteins, lipids, carbohydrates and nucleic acids. The majority of free radicals that damage biological systems are oxygen-free radicals, and these are more generally known as "reactive oxygen species" (ROS). These are the main byproducts formed in the cells of aerobic organisms, and can initiate autocatalytic reactions so that molecules to which they react are themselves converted into free radicals to propagate the chain of damage. ROS can be (i) generated during UV light irradiation and by X-rays and gamma rays (ii) produced during metal catalyzed reactions (iii) are present in the atmosphere as pollutants (iv) are produced by neutrophils and macrophages during inflammation and (iv) are by-products of mitochondrial catalyzed electron transport reactions, and various other mechanisms (Cadenas,1989).

The amount of free radical production is determined by the balance of many factors, and ROS are produced both endogenously and exogenously (Inoue *et al.,* 2003). The endogenous sources of ROS include mitochondria, cytochrome P_{450} metabolism, peroxisomes and inflammatory cell activation (Inoue *et al.,* 2003). Examples of oxygen-derived free radicals include superoxide radical ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$). Hydrogen peroxide, although not a radical species is produced in the mitochondria as is its ROS precursor superoxide. It has been proposed that ubisemiquinone is the main reductant of oxygen in mitochondrial membranes and

the generation of superoxide within mitochondria is approximately 2–3 nmol/min per mg of protein (Inoue *et al.,* 2003). The presence of ubiquinone indicates it to be the most important physiological source of this radical in living organisms (Inoue *et al.,* 2003). Since mitochondria are the major site of free radical generation, they contain a variety of antioxidants including enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) which are present on both sides of their membranes in order to minimize ROS induced stress (Cadenas and Davies 2000). There are also other cellular sources of superoxide radicals present such as the enzyme xanthine oxidase, which catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion followed by the generation of hydrogen peroxide (Valko *et al.,* 2004). ROS can also be produced by a host of exogenous sources such as xenobiotics, chlorinated compounds, environmental agents, metals (redox and non-redox), ions, and radiation (Valko *et al.,* 2006).

ROS can be both harmful and beneficial in biological systems depending on the environment (Lopaczynski and Zeisel 2001; Glade 2003). Beneficial effects of ROS involve, for example, the physiological roles in cellular responses to noxia such as defense against infectious agents, and in the function of a number of cellular signaling systems. In contrast, at high concentrations, ROS can mediate damage to cell structures, including lipid membranes, proteins and nucleic acids, and this damage is often referred to as "oxidative stress" (Poli *et al* 2004). The harmful effects of these free radicals (ROS) have been found in children with severe PEM which is responsible for cell damage leading to oedema, fatty liver and skin lesions (Golden *et al.,* 1990), and are balanced by the action of antioxidants, some of which are enzymes present in the body (Halliwell, 1996). Despite the presence of the cell's antioxidant defense system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle and has been

implicated in aging and diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions (Rahman, 2003). Antioxidants neutralize these damaging free radicals by quenching the reactive molecules, thereby protecting cells from endogenous and exogenous stressors and prolonging their life and vitality (Halliwell, 1996) .

2.7 Antioxidants

Antioxidant is a substance that when present at low concentration compared to that of an oxidizable substrate, would significantly delay or prevent oxidation of that substrate (Halliwell, 1995). The oxidizable substrate includes almost everything found in living cells including carbohydrates, DNA, lipids and proteins (Lequeux *et al.*, 2010). Antioxidants are a conspicuous first line of defense against free radicals and are able to counteract or at least slow all major steps in the process of free radical induction of cancer (Rahman, 2003).

Antioxidants are broadly divided into enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants include the superoxide dismutase, glutathione peroxidase and catalase (Klaunig and Kamendulis, 2004). Superoxide dismutase and glutathione peroxidase, which are present in cytosol and mitochondria, reduce the superoxide anion to hydrogen peroxide and water, and remove the majority of hydrogen peroxide, respectively, while, catalase located in the peroxisomes, also removes high levels of hydrogen peroxide (Fridovich, 1986). Non-enzymatic antioxidants which include vitamin A, C, E, trace elements such as zinc, copper and selenium, reduced glutathione, and coenzyme Q function to quench reactive oxygen species (Clarkson and Thompson 2000).

These antioxidant enzymes are synthesized by the body to keep the concentration of the free radicals at a non-harmful level but the trace elements (Selenium, Zinc, and Copper) needed as cofactors must be supplied by the diet. Selenium (Se) is a cofactor for glutathione peroxidase

(Chan *et al.*, 1998). The superoxide dismutase present in the cytosol contains zinc (Zn) and copper (Cu) (Fridovich, 1986).

2.7.1 The need for antioxidants defense

Most biologically relevant free radicals are derived from oxygen and nitrogen and the so-called ROS and Reactive Nitrogen Species (RNS) (Peter, 2007). Both these elements are essential but in certain circumstances are converted into free radicals which are highly unstable and their reactive capacity makes them capable of damaging biologically relevant molecules such as proteins, lipid or carbohydrates (Peter, 2007). Antioxidants are molecules that retard or prevent the oxidation of other compounds (Halliwell, 1995). Not only soluble antioxidants but also complex enzymatic systems such as catalase, superoxide dismutase and some peroxidases may be used by cells to avoid undesired oxidations (Angelo, 2009). Sarvajeet and Narendra (2010) reported that the ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins and causing lipid peroxidation. it is important to note that whether ROS will act as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time. Oxidative stress occurs when this critical balance is disrupted due to depletion of antioxidants or excess accumulation of ROS, or both (Scandalios, 2005).

2.7.2 Mechanisms of antioxidant functions

Antioxidants are effective through various mechanisms including: 1) preventive antioxidants, 2) free radical scavengers, 3) sequestration of elements by chelation and

4) quenching active oxygen species (McDowell *et al.*, 2007).

Preventive antioxidants:

These antioxidants suppress formation of free radicals; for example catalase (Fe containing) and glutathione peroxidase (Se containing), two antioxidant enzymes, decompose hydrogen peroxide, preventing the formation of oxygen radicals (McDowell *et al.,* 2007).

Free radical scavengers:

These antioxidants confer stability to the 'reactive' species by donation of an electron and become oxidized themselves to form a more stable radical. For example α-tocopherol (vitamin E) scavenges peroxyl radicals and is converted to a tocopherol radical. Illustrating antioxidant interactions, the vitamin E becomes "reactivated" by ascorbic acid donating an electron which in turn forms an ascorbate radical in the process (McDowell *et al.,* 2007).

Sequestration of metal by chelation:

Although trace minerals are important dietary constituents, they can act as pro-oxidants (promote free radical formation). Since trace minerals such as Fe and Cu can propagate the formation of more reactive radicals they are kept bound to transport proteins such as transferrin or ceruloplasmin, as this renders them less available to contribute to radical or pro-oxidant formation (McDowell *et al.,* 2007).

Quenching of active oxygen species:

Antioxidants can convert active oxygen species to more stable forms, for example, superoxide dismutase (Zn and Cu containing), carotenoids and vitamin E stabilize singlet oxygen radicals, forming less reactive hydrogen peroxide (McDowell *et al.,* 2007).

2.7.3 Enzymatic antioxidants and their cofactors

Glutathione peroxidase

There are two forms of this enzyme, one which is selenium-dependent and the other, which is selenium-independent (glutathione-S-transferase, GST) (Mates *et al.*, 1999). The differences are due to the number of subunits, catalytic mechanism and the bonding of selenium at the active centre, and glutathione metabolism is one of the most important antioxidative defense mechanisms present in the cells. There are four different Se-dependent glutathione peroxidases present in humans (Chaudière and Ferrari-Iliou, 1999) and these are known to add two electrons to reduce peroxides by forming selenoles (Se-OH) 9and the antioxidant properties of these seleno-enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. Selenium-dependent glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also competes with catalase for hydrogen peroxide as a substrate and is the major source of protection against low levels of oxidative stress (Chaudière and Ferrari-Iliou 1999).

Catalase

This enzyme is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen (Mates *et al.*, 1999). Catalase has one of the highest turnover rates of all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen every minute (Mates *et al.*, 1999).

Superoxide dismutase (SOD)

This is one of the most effective intracellular enzymatic antioxidants and it catalyzes the conversion of superoxide anions to dioxygen and hydrogen peroxide (Landis and Tower 2005). Superoxide dismutase exists in several iso-forms, which differ in the nature of active metal centre, amino acid composition, co-factors and other features (Landis and Tower 2005). There are three forms of SOD present in humans: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extra cellular-SOD (Landis and Tower 2005). Superoxide dismutase neutralizes superoxide ions by going through successive oxidative and reductive cycles of transition metal ions at its active site (Chaudière and Ferrari-Iliou 1999). Cu, Zn-SOD has two identical subunits with a molecular weight of 32 kDa (Mates *et al.*, 1999) and each of the subunit contains as the active site, a dinulcear metal cluster constituted by copper and zinc ions, and it specifically catalyzes the dismutation of the superoxide anion to oxygen and water (Mates *et al.*, 1999). The mitochondrial Mn-SOD is a homotetramer with a molecular weight of 96 kDa and contains one manganese atom per subunit (Mates *et al.*, 1999), and it cycles from Mn(III) to Mn(II) and back to Mn(III) during the two-step dismutation of superoxide. Extracellular superoxide dismutase contains copper and zinc, and is a tetrameric glycoprotein having a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate (Mates *et al.*, 1999). However, its regulation in mammalian tissues occurs primarily in a manner coordinated by cytokines, rather than as a response to oxidative stress (Rahman, 2007).

2.7.4 Cofactors

The biochemical definition of a co-factor is that it is an ion or a molecule that binds to the catalytic site of an apoenzyme rendering it active (Rahman, 2007). Many enzymes require metal

ions for their activity and these metal ions are also referred to as co-factors. The major antioxidant enzymes possess trace elements or metals at the catalytic site and the availability of these cofactors can determine the activity of such enzymes (Rahman, 2007).

Different essential metals play an important role in controlling oxidative reactions in biological tissues. For example, Cu is an essential cofactor in a number of critical enzymes including cytochrome C oxidase and copper, zinc-superoxide dismutase (Cu, Zn-SOD) (Arredondo and Núñez, 2005). Although unregulated Cu is also a well known pro-oxidant it can through the action of transporter proteins such as metallothineins and ceruplasmin exert its antioxidative effects (Leung, 1998). A Cu deficiency-induced decrease in the activity of Cu, Zn-SOD in humans and animals has been reported (Turnlund *et al.*, 1997; Uriu-Adams *et al.*, 2005). Copper deficiency also decreases the activity of ceruloplasmin, which requires Cu for its ferroxidase function (Hellman and Gitilin, 2002), and it can also lead to a reduction in enzymes of the oxidant defense system such as selenium-dependent glutathione peroxidase (Se-GPX) and catalase (Chen *et al.*, 1994). Furthermore a deficiency in Cu can also alter other ROS scavengers including metallothionein (a Cu and Zn containing protein) (Tapia *et al.*, 2004) and the non-protein thiol, glutathione (Uriu-Adams and Keen, 2005). Copper and zinc are also essential co-factors for enzymes involved in the synthesis of various bone matrix constituents and could be important in the elderly since they may play an important role in reducing bone loss in osteoporosis (Lowe *et al.*, 2002).

Iron is an essential constituent of catalase enzymes, haemoglobin and myoglobin, but is also a pro-oxidant (through Fenton reactions) when it is present in excess (Gutteridge, 1995; Leung, 1998; Puntarulo, 2005). In the presence of lipids, iron creates oxidative stress and subjects with

high levels of lipids and serum iron are at an increased risk of cancer (Mainous *et al.*, 2005). Thus, iron chelators such as albumin, haptoglobin, lactoferrin, transferrin and urate also have an important role to play in preventing oxidative stress-related diseases (Gutteridge, 1995).

Selenium is another important co-factor and epidemiological findings have linked a lowered selenium status to neurodegenerative and cardiovascular diseases as well as to an increased risk of cancer (Brenneisen *et al.*, 2005). There is an association between Se reduction, DNA damage and oxidative stress, and there is some evidence that Se may affect not only cancer risk but also progression and metastasis (Rayman 2005). Selenium intervention in subjects with a lower Se status has shown some benefits in reducing the incidence and mortality in all cancers but more specifically in liver, prostate, colo-rectal and lung cancers. Its protective effects appear to be associated with its presence in multiforms of glutathione peroxidases, which are known to protect DNA and other cellular damage from oxidative stress (Schrauzer,2000; Trueba *et al.*, 2004).

The element manganese is another co-factor involved in antioxidant defense mechanisms and is a vital component of Mn-SOD enzyme, which plays a crucial role in protecting mitochondria from free radical attack (Leung, 1998).

Zinc is another component of SOD which is also involved in antioxidant defense systems and protects the vascular and immunological systems from the damaging effect of free radical species (Kuppusamy *et al.*, 2005). Zinc is also a key constituent or co-factor of over 300 mammalian proteins which may have a role in the prevention of initiation and progression of cancer (Kuppusamy *et al.*, 2005). Evidence supports the fact that Zn deficiency can impair the host protective mechanisms designed to protect against DNA damage, thus increasing the risk of

cancer (Ho, 2004), and it also plays an important role as an antioxidant and/or as a co-factor in keeping the skin healthy (Rostan *et al.*, 2002).

2.7.5 Non-enzymatic antioxidants

In addition to trace elements that serve as cofactors of antioxidative enzymes, diets also provide antioxidant vitamins (vitamin A, C and E) that react directly with free radicals in a non catalytic manner (Halliwell, 1995).

Vitamin A

Many hundreds of carotenoids are found in nature but relatively few are found in human tissues, the five main ones being β-carotene, lutein, lycopene, β-cryptoxanthin, and α-carotene (Thurnham, 1994; Rock *et al.,* 1996). β-Carotene is the main source of pro-vitamin A in the diet (Mortensen *et al.*, 2001). There are approximately 50 carotenoids with pro-vitamin A activity, but β-carotene is the most important and is one of the most widely distributed carotenoids in plant species (Mortensen *et al.*, 2001). Carotenoids contain conjugated double bonds and their antioxidant activity arises due to the ability of these carotenoids to delocalize unpaired electrons (Mortensen *et al.*, 2001). This is also responsible for the ability of carotenoids to physically quench singlet oxygen without degradation and for the chemical reactivity of carotenoids with free radicals. The efficacy of carotenoids for physical quenching is related to the number of conjugated double bonds present in the molecule, which determines their lowest triplet energy level (Stahl and Sies, 2003). They can also scavenge peroxy radical, thus preventing damage in lipophilic compartments (Stahl and Sies, 2003). The concentrations of carotenoids and the partial pressure of oxygen are also important factors in their effectiveness as antioxidants. Carotenoids,

in particular β-carotene exhibit antioxidant properties at low oxygen partial pressure but become a pro-oxidant at high pressures of oxygen and similarly, at high carotenoid concentrations, pro-oxidant action is displayed (Rice-Evans *et al.*, 1997; Stahl and Sies, 2003).

Diet also contains non-nutrients compounds that may have antioxidative effects. For instance, phenolic and polyphenolic substances like flavonoids and catechin are potent antioxidants (Fang *et al.,* 2002). Flavonoids are able to scavenge superoxide, hydroxyl radicals and peroxyl radicals. (Fang *et al.,* 2002).

Vitamin C (ascorbic acid)

This is an important and powerful water-soluble antioxidant and thus works in aqueous environments of the body (Carr and Frei, 1999; Kojo, 2004). Its primary antioxidant partners are vitamin E and the carotenoids, it also works along with the antioxidant enzymes (Carr and Frei, 1999; Kojo, 2004). Vitamin C acts with vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (Carr and Frei, 1999; Kojo, 2004), and also raises intracellular glutathione levels thus playing an important role in protein thiol group protection against oxidation (Naziroglu and Butterworth 2005).

Vitamin E

This is a fat-soluble vitamin of plant origin existing in eight different forms (Hensley *et al.*, 2004). In humans, α-tocopherol is the most active form, and is the most powerful membrane bound antioxidant employed by the cell (Hensley *et al.*, 2004). The main function of vitamin E is to protect against lipid peroxidation (Pryor, 2000). Membrane lipids are susceptible to chain reaction initiated by free radicals, in which lipids peroxyl radicals react with other lipid

molecules to form new lipid radicals. Vitamin E acts as a chain breaking antioxidant, since it is able to prevent or slow down the propagation of free radical reactions in the cell membranes (Halliwell, 1996; Devasagayam *et al.,* 2004). Vitamin E also quenches singlet oxygen and reacts with peroxynitrite (Stahl and Sies, 1997).

2.7.6 Oxidative Stress

Oxidative stress in biological systems results from the overproduction of reactive oxygen species or decrease in antioxidant potential (Sies, 1985). Depletions of enzymatic and non-enzymatic antioxidants and other essential constituents (such as copper, zinc, iron, magnesium and selenium) can also cause oxidative stress (Sies, 1985). Oxidative stress can also be induced by variety of factors such as radiation or exposure to heavy metals and xenobiotics (example carbon tetrachloride) (Kim *et al.,* 1990). It may be due to the presence of toxins that produce ROS (example paraquat), or activation of natural system that produce ROS, such as inappropriate activation of phagocytes (Halliwell and Gutteridge, 2006). Several cell types respond to mild oxidative stress by proliferation, which can be good to wound healing but bad if it leads to tissue fibrosis (Cave *et al.,* 2005). Cells may adapt to the stress by up-regulating defense and/or repair systems. This may protect against damage to some extent (but not completely) or sometimes confer overprotection. The cells are then resistant to higher levels of oxidative stress imposed subsequently. Under physiological conditions, about 95% of oxygen consumed is reduced to water by cytochrome oxidase in the mitochondria, but the remaining 5% is released as ROS especially as superoxide anion and hydrogen peroxide (Buonocore *et al.,* 2010).

Harmful levels of these reactive oxygen species (free radicals) have been found in children with PEM which result in oxidative stress leading to oedema, skin lesion and fatty liver (Golden *et al.,*

1990). Oxidative stress has also been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes mellitus, ischemia and aging (Dalle-Donne *et al.,* 2006).

2.8 Roles of Trace Elements in Nutrition

Trace elements are nutrients required by the body in minute quantities to maintain proper functioning (Tapiero and Tew, 2003). Although required in very small amounts, trace elements such as zinc, copper, selenium, iron, iodine, manganese, fluoride, chromium, and molybdenum are vital for maintaining health. Also referred to as micro minerals, these trace elements are part of enzymes, hormones and cells in the body. Insufficient intake of trace minerals can cause symptoms of nutritional deficiency. However, needs for these trace elements are easily met by eating a variety of foods from the different food groups (Tapiero and Tew, 2003).

Zinc

Zinc supports the function of numerous proteins in the body, such as the metalloenzymes, which are involved in variety of metabolic processes, including the regulation of gene expression (Tapiero and Tew, 2003). In addition, zinc stabilizes cell membranes, helping to strengthen their defense against free-radical attack (Kuppusamy *et al*., 2005). It assists in immune function, growth and development, interacts with platelets in blood clotting, affects thyroid hormone function, and influences behavior and learning performances. It is also essential to normal taste perception, wound healing, sperm synthesis, and fetal development (Tapiero and Tew, 2003). Zinc deficiency impairs all these and other functions, underlining the vast importance of zinc in supporting the body's proteins. The highest source of zinc is found in protein-rich foods such as shellfish, meats, poultry, milk and cheese. Legumes and whole-grain products are good sources

of zinc if consumed in large quantities. Dietary factors can influence zinc absorption. For example, phytates binds zinc, thus limiting its bioavailability (Adams *et al.,* 2002).

Copper

Copper serves as a constituent of several enzymes. Its role in iron metabolism makes it a key factor in haemoglobin synthesis. Copper and zinc-containing enzymes participate in the body natural defense against free radicals. It helps to manufacture collagen and heal wounds. The richest sources of copper are legumes, whole grains, nuts, shellfish and seeds. Over half of the copper from food is absorbed, and the major route of elimination appears is the bile. Water may also provide copper, depending on type of plumbing pipe and the hardness of the water (Hellman and Gitlin, 2002).

Selenium

Selenium is one of the body's antioxidant nutrients, working primarily as a part of the enzyme glutathione peroxidase which prevents free-radical formation, thus blocking the chain reaction before it begins (Burk and Hill, 2005). Selenium from food appears to be more effective in inhibiting cancer growth than selenium from supplements (Duffield-Lillico *et al.,* 2004). Selenium is found in the soil, and therefore in the crops grown for consumption (Finley, 2005).

Iron

Iron is an essential nutrient, vital to many of the cell's activities; it serves as a cofactor to enzymes involved in oxidation-reduction reactions (Gutteridge, 1995). Enzymes involved in making amino acids, collagen, hormones, and neurotransmitters all require iron. Most of the body's iron is found in two proteins: hemoglobin in the red blood cells and myoglobin in the muscle cells. Meat, fish, and poultry contribute the most iron per serving. Other protein-rich food such as legumes and eggs are also good sources (Hentze *et al.,* 2004).

Iodine

Iodine is an integral part of the thyroid hormones that regulate body temperature, metabolic rate, reproduction, growth, blood cell production, nerve and muscle function. By controlling the rate at which the cells use oxygen, these hormones influence the amount of energy released during basal metabolism. The ocean is the world's major source of iodine. In coastal area's, seafood, water and even iodine-containing sea mist are dependable iodine sources (Zimmerman, 2006).

Manganese

Manganese acts as a cofactor for many enzymes that facilitate the metabolism of carbohydrate, lipids and amino acids. In addition, manganese-containing metalloenzymes assist in bone formation. Grain products make the greatest contribution of manganese to the diet (Finley, 2005).

Fluoride

Fluoride is present in virtually all the soils, water supplies, plants and animals. Only a trace of fluoride occurs in the human body, but with this amount, the crystalline deposits in bones and teeth are larger and more perfectly formed to increase hardness of teeth, stronger bones and prevent dental caries (Finley, 2005).

Chromium

Chromium is an essential mineral that participates in carbohydrate and lipid metabolism. It helps to maintain homeostasis by enhancing the activity of the hormone insulin. When chromium is lacking, a diabetes-like condition may develop with elevated blood glucose and impaired glucose

tolerance, insulin response, and glucagon response. The best sources of chromium are unrefined foods, particularly liver, brewer's yeast and whole grain (Althuis *et al.,* 2002).

Molybdenum

Molybdenum helps to activate several metalloenzymes and enables normal cell function. Dietary sources of molybdenum include milk, legumes, whole-grain breads and nuts (Finley, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study location

The study was conducted in the out-Patient clinics of the Department of Paediatrics, Ahmadu Bello University Teaching Hospital (ABUTH), Shika-Zaria and Institute of Child Health (ICH), Banzazzau, Zaria. Zaria is situated in Kaduna State, northern Nigeria. It is one of the agricultural areas with the majority of the population being Hausas. It has an area of 33,000 sq km and a population of about 406,990 (Musa *et al.*, 2009).

3.1.2 Study population

A total of 98 children with PEM were recruited for this study and they were age- and sex-matched with apparently healthy well nourished under-five children as controls.

3.1.3 Inclusion criteria for children with PEM

i. All children less than 5 years being seen at the out-patient clinics of the Department of Paediatrics.
ii. All children with PEM, such as children whose weight was 60-80% of expected weight for age with the presence of oedema were classified as kwashiorkor. Children whose weight was less than 60% of expected weight for age with the absence of oedema were classified as marasmic. Children whose weight was less than 60% of expected weight for age with the presence of oedema were classified as marasmic-kwashiorkor (Wellcome, 1970).

3.1.4 Inclusion criteria for controls

i. Apparently healthy well nourished under-five children without any features suggestive of PEM attending the same clinics whose weight was greater than 80% of expected weight for age without oedema.

3.1.5 Exclusion criteria

i. All children already receiving food supplements.

ii. All children that were not in stable conditions such as children that were having fever and those with severe skin lesions.

3.1.6 Informed consent

Informed consent for inclusion into this study was sought from the parents/guardians of the selected children using standard protocol (Appendices I and II).

3.1.7 Ethical approval

Ethical approval was obtained from the Ethical Committee of ABUTH, Shika-Zaria, in accordance with Helsinki declaration. This is a code of ethics on human experimentation drafted by the World Medical Association in 1964.

3.1.8 Sample size determination

Determination of the sample size for the study was done using standard formula for calculation of minimum sample size by Singha, (1996) and Oyejide, (1992).

$$\text{Sample size (n)} = \frac{(Z_1 - a)^2 - (P)(1 - P)}{d^2}$$

Where n = minimum sample size; $Z_1 - a$ = the value of standard normal deviation which at 95% confident interval has been found to be 1.96; P = the estimate of population prevalence; d = the difference between the true population rate and sample that can be tolerated (precision).

In Kaduna state, a malnutrition prevalence of 6.8% was reported in Zaria (Alegbejo and Yakubu, 1993). Therefore, at 6.8% prevalence of PEM, using 5% precision at 95% confident interval, the minimum sample size n for this study was calculated as follows:

$$n = \frac{(1.96)^2 (0.068)(1 - 0.068)}{(0.05)^2}$$

$$\frac{3.84 \times 0.068 \times 0.932}{0.0025}$$

$$97.3 \approx 97$$

The minimum sample size of children with PEM was 97 as calculated. Therefore, 98 children with PEM were selected in this study using one as attrition. Similarly, 98 age- and sex -matched well nourished children without PEM were also selected as controls.

3.1.9 Sampling techniques

A simple random sampling technique was used to select children with PEM from the population of patients attending Paediatrics out-patients clinics, ABUTH, Zaria. Arrangements were made with the clinicians whereby children who satisfied the study inclusion criteria were selected.

Relevant medical information was obtained from the parents/guardians of the selected child and filled into the study proforma (Appendix III).

3.1.10 Blood sample collection

Two milliliters of blood were collected from peripheral vein by venipuncture using a sterilized syringe. This procedure was carried out by the paediatricians. This was performed using a syringe and needle after cleaning the site with methylated spirit. In this case, the antecubital fossa was cleaned with methylated spirit and allowed to dry. A tourniquet was applied a few centimeters above the antecubital fossa to distend the veins, after which blood was collected using a sterilized 5ml syringe and 23 G needle. The blood was placed in sample bottles and allowed 30-60 minutes for spontaneous blood clotting. The serum was separated from the blood cells by centrifugation at 4000 rpm for 5 minutes at room temperature, and decanted using a Pasteur pipette and used for the assays.

3.1.11 Chemicals

All assay kits were procured from Northwest Life Science Specialties Vancouver, Canada. The chemicals and reagents used for this study were procured from Sigma Chemical Company St. Louis, U.S.A and were of analytical grade.

3.1.12 Equipment

Hettich Universal Centrifuge was used to spin the blood specimens. Ultra-Spec plus Spectrophotometer was used for the measurements of serum total protein, albumin, superoxide dismutase and glutathione peroxidase. Atomic absorption spectrophotometer (Model- AA240FS) was used for the measurements of serum zinc and copper.

3.2 Methods

3.2.1 Questionnaire

Semi-structured questionnaire was used to obtain data on demographic and socio-economic status of patients with PEM (Appendix III). Classification of the different social classes was explained in the proforma (Appendix IV).

3.2.2 Measurements of biochemical parameters

3.2.2.1 Serum total protein

Serum total protein levels were determined using Biuret method as described by Reinhold (1953).

Principle

Cupric ions (Cu^{2+}) in the biuret reagent complex with NH_3^+ groups involved in the peptide bonds. In the presence of alkaline media (3% NaOH) and at least two peptide bonds a violet coloured chelate was formed. The absorbance of the coloured chelate was measured at 540nm using spectrophotometer.

Procedure

The Biuret reagent (2.5ml) was pipetted into three test tubes labeled as blank, standard and test. Sample (0.05ml) was pipetted into the test and 0.05ml of standard reagent was pipette into the standard. The contents of the three test tubes were properly mixed and incubated for 10 minutes at room temperature before measuring the absorbance of test and standard at 540 nm against reagent blank using 1 cm light path cuvette. The total protein concentration in the sample was calculated using the formula below:

Total Protein Concentration (gm/dl) = Absorbance of Test x Standard Concentration

Absorbance of Standard

3.2.2.2 Serum albumin

Serum albumin levels were determined by the bromocresolgreen (BCG) method as described by Hill (1985).

Principle

Albumin reacts with the dye –bromocresol green which has specific affinity for it in acidic solutions to produce a coloured complex which is measured colorimetrically at 630nm. The absorbance is directly proportional to the concentration of albumin in the sample.

Procedure

The BCG reagent (2.5ml) was pipetted into three test tubes labeled as blank, standard and test. The serum (0.01ml) was pipetted into the test and 0.01ml of the standard was pipetted into the standard. The contents of the three test tubes were properly mixed and incubated for 10 minutes at room temperature (not less than 25°C), then the absorbance of test and standard was measured against reagent blank at 630 nm after the incubation using 1 cm light path cuvette.

The albumin concentration in the sample was calculated using the formula:

Albumin Concentration (gm/dl) = Absorbance of Test x Standard Concentration

Absorbance of Standard

3.2.2.3 Serum zinc and copper

Serum zinc and copper were determined using atomic absorption spectrophotometer (Model No. AA240FS), Varian, Sweden.

Principle

The principle of atomic absorption spectrophotometer is based on the measurement of resonance radiant energy absorbed by unexcited radiant energy (ground state) of free atoms in a sample cell. The population of the free atoms (the concentration) is directly proportional to the degree of the absorption.

Procedure

Two hundred and fifty microlitres (250µl) of sample were removed from the original sample and digested with 2ml trace metal grade concentrated HNO_3. The sample was then heated to 100^oC for two hours and allowed to cool at room temperature. Once complete digestion was achieved, 300µl of 30% H_2O_2 were added to each sample followed by heat-instilling until dry. The sample was then reconstituted to 10ml with 0.5% HNO_3 in deionized water, and the elements analyzed using atomic absorption spectrophotometer.

3.2.2.4 Serum superoxide dismutase

Serum superoxide dismutase (SOD) was determined using colorimetric method as described by Martin *et al.*, (1987).

Principle

Auto-oxidation of haematoxylin (with increase in absorbance at 560 nm) is inhibited by SOD activity at the assay pH 7.8; the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. SOD activity in the sample is determined by measuring the amount of haematin at 560 nm.

Haematoxylin + $O_2 \rightarrow H_2O_2$ + Haematin (active form of haematoxylin)

Auto-oxidation

Procedure

Sample and standard layout were recorded. To each well used for testing, 230 µL of assay buffer were added with 10 µL of assay buffer (for blank) or 10 µL sample. The mixture was shaken and incubated for 2 minutes before adding 10 µL of hematoxylin reagent to begin reaction using a multi-channel pipette. The reaction was quickly mixed using the instrument's shaker function and the absorbance at 560 nm every 60 seconds or smaller time interval for at least 5 minutes was immediately recorded.

3.2.2.5 Serum glutathione peroxidase

Serum glutathione peroxidase (GPx) was determined using colorimeteric method as described by Paglia and Valentine (1967).

Principle

Glutathione peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$ which results in decreased absorbance at 340nm and recycling the GSH. Glutathione peroxidase activity is determined after taking absorbance at 340nm.

Procedure

All reagents were brought to room temperature. Microplates were removed from plastic bag and 50 µL of diluted sample (or controls if present) were added to the wells, before adding 50 µL of working NADPH solution and 50 µL of working H_2O_2 solution to each well. After 1 minute, absorbance was monitored at 340nm for 5 minutes with a recording interval of 60 seconds and Glutathione peroxidase activity was calculated from the calibration curve.

3.2.3 Statistical analysis

Statistical analysis was performed using SPSS programme (version 20.0 SPSS Incorporation, Chicago, IL, USA). Results obtained were expressed as mean ± standard deviation. The results obtained from the questionnaire were subjected to descriptive statistics. The data obtained from protein-energy malnourished children and controls were compared using student's t-test. The results obtained from different classes of protein-energy malnourished children and controls were compared using one way analysis of variance (ANOVA). The differences between the various means were compared using Duncan Multiple Range Test. Pearson correlation was used to study the relationship between the trace elements and antioxidant enzymes in both malnourished patients and controls. P-value of less than 0.05 ($p<0.05$) was considered as significant.

CHAPTER FOUR

RESULTS

Findings of the study are presented in Tables 4.1- 4.7. Data are presented as means and standard deviation, as well as correlation coefficients.

4.1 Characteristics of study groups

The demographic and socio-economic characteristics of the study groups are presented in Table 4.1. The patients and controls had the mean (±SD) age (months) of 18.54±7.10 and 18.41±9.93 respectively. Majority of the children were in the age group 12- 23 months. Most of the PEM patients and controls were Hausas (89.71%) and (70.41%) respectively. The highest proportions of the PEM patients were from parents with lower economic and educational status (72.45%), while the controls were from parents with higher economic and educational status (67.26%).

4.2: Feeding characteristics of PEM patients

Feeding characteristics of PEM patients are presented in Table 4.2. The results show that majority of the patients with PEM and controls were not exclusively breast-fed (76.53%) and (63.27%) respectively. Although, the practice of exclusive breast-feeding was lower in the PEM patients than in the controls. However, less than half of the PEM patient and controls were still breast-feeding (38.78%) and (44.81%) respectively.

Table 4.1: Demographic and Socio-economic Characteristics of Study Groups

Characteristics	PEM (n= 98)	Percentage (%)	Controls (n= 98)	Percentage (%)
Age group (months)				
1- 11	14	14.29	30	30.61
12- 23	59	60.20	35	35.71
24- 35	22	22.45	26	26.53
36- 47	2	2.04	5	5.10
48- 59	1	1.02	2	2.04
Sex				
Males	57	58.16	57	58.16
Female	41	41.84	41	41.84
Ethnicity				
Hausa	88	89.71	69	70.41
Yoruba	0	0.00	5	5.10
Others	10	10.20	24	24.49
Social class				
I	1	1.02	27	27.55
II	8	8.16	39	39.71
III	18	18.37	16	16.33
IV	34	34.69	8	8.16
V	37	37.76	8	8.16

Social class: refers to group of people within a society who possesses the same economic and social position based on education, income and occupation. Social class I and II are people from higher socio-economic status, III and IV are people from lower socio-economic status. Social class V are people from with the least socio-economic status.

4.2: Feeding Characteristics of Children with PEM

Characteristics	PEM (n= 98)	Percentage (%)	Controls (n= 98)	Percentage (%)
Exclusively breastfed children				
Yes	23	23.47	36	36.73
No	75	76.53	62	63.27
Weaning period				
≤ 5months	5	5.10	1	1.02
6-11	9	9.18	2	2.04
12-17	20	20.41	24	24.49
≥ 18	24	24.49	27	27.55
Still breast feeding	38	38.78	44	44.81
No breast feeding	2	2.04	0	0.00

PEM = Protein-Energy Malnutrition.

4.3: Serum biochemical parameters of PEM patients

Serum biochemical parameters in PEM patients are shown in Table 4.3, which indicates that the total protein, albumin, SOD and GPX were significantly lower ($p<0.05$) in PEM patients than control, while zinc concentrations in PEM patients were significantly higher ($p<0.05$) than in controls.

4.4 Serum total protein and albumin levels in classes of PEM patients

Table 4.4 presents the levels of serum total protein and albumin in different classes of PEM patients. The results show that the serum total protein and albumin levels in marasmus, kwashiorkor and marasmic-kwashiorkor were significantly lower ($p<0.05$) than in controls.

However, there was no significant difference ($p<0.05$) in serum total protein and albumin levels between kwashiorkor and marasmic-kwashiorkor, but there was a significant difference ($p<0.05$) in serum total protein and albumin levels between marasmus with kwashiorkor and marasmic-kwashiorkor.

4.5 Serum concentrations of some trace elements in PEM patients

Serum zinc and copper concentrations in different classes of children with PEM are presented in Table 4.5. The serum zinc concentrations in marasmus, kwashiorkor and marasmic-kwashiorkor were significantly higher ($p<0.05$) than in controls. However, there was no significant difference ($p<0.05$) in serum copper concentration in marasmus, kwashiorkor and marasmic-kwashiorkor compared with than of the controls, but serum copper concentration was significantly higher ($p<0.05$) in kwashiorkor compared with that of the marasmic-kwashiorkor.

4.6 Serum levels of some antioxidant enzymes in PEM patients

Serum levels of superoxide dismutase and glutathione peroxidase in different classes of children with PEM are presented in Table 4.6. The results show that superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels were significantly lower ($p<0.05$) in marasmus, kwashiorkor and marasmic-kwashiorkor compared with that of the controls. However, there was no significant difference ($p<0.05$) in the levels of these enzymes in children with kwashiorkor and those with marasmic-kwashiorkor.

Table 4.3: Serum Biochemical Parameters of Children with PEM

Subjects	n	Total Protein(gm/dl)	Albumin (gm/dl)	Zinc (mg/l)	Copper (mg/l)	SOD (U/l)	GPX (U/l)
PEM Children	98	55.76±3.95[a]	26.43±2.78[a]	8.37±4.25[b]	2.40±1.12[a]	1.87±0.32[a]	42.38±5.03[a]
Controls	98	64.72±2.87[b]	37.46±3.36[b]	5.14±2.39[a]	2.82±1.18[a]	2.41±0.22[b]	58.78±3.98[b]

Values are Mean±SD. Values with different superscripts down the column are significantly different ($p<0.05$), n = sample size, SOD = Superoxide dismutase, GPx = Glutathione peroxidase, PEM = Protein-Energy Malnutrition.

Table 4.4: Serum Total Protein and Albumin Levels in Different Classes of Children with PEM

Groups	Total Protein (gm/dl)	Albumin (gm/dl)
Marasmus	56.57± 3.54[b]	26.92± 2.62[b]
Kwashiorkor	51.20± 2.95[a]	23.00±2.55[a]
Marasmic-kwashiorkor	51.30± 3.30[a]	24.10± 1.73[a]
Controls	64.72± 2.87[c]	37.46± 3.36[c]

Values are means ± SD. values with different superscripts are significantly different ($p<0.05$) down the column.

Table 4.5: Serum Zinc and Copper Concentration in Different Classes of Children with PEM

Groups	Zinc (mg/l)	Copper (mg/l)
Marasmus	8.32± 4.32[b]	2.73± 0.94[ab]
Kwashiorkor	9.36± 1.76[b]	3.76± 2.49[b]
Marasmic-kwashiorkor	8.30± 4.77[b]	1.96± 1.04[a]
Controls	5.14± 2.39[a]	2.82± 1.18[ab]

Values are means ± SD. Values with different superscripts are significantly different ($p<0.05$) down the column.

Table 4.6: Serum Superoxide Dismutase and Glutathione Peroxidase Levels in Different Classes of Children with PEM

Groups	SOD (U/l)	GPx (U/l)
Marasmus	1.92± 0.21[b]	43.28± 4.48[b]
Kwashiorkor	1.72± 0.13[ab]	35.40± 4.72[a]
Marasmic-kwashiorkor	1.54± 0.32[a]	38.40± 5.25[a]
Controls	2.14± 0.22[c]	58.78± 3.98[c]

Values are means ± SD. Values with different superscripts are significantly different ($p<0.05$) down the column. SOD = Superoxide dismutase, GPx = Glutathione peroxidase.

4.7 Pearson's Correlation between some Trace Elements (Cu, Zn,) and Antioxidant Enzymes (SOD, GPx) in Children with PEM

Pearson's correlation between serum concentrations of zinc and copper with serum levels of SOD and GPx in different classes of PEM patients are presented in Table 4.7. There were positive correlations between serum zinc and copper with SOD in both marasmus and controls but not in kwashiorkor and marasmic-kwashiorkor. However, GPx activities are negatively correlated with both zinc and copper in almost all the groups except copper in marasmus.

Table 4.7: Pearson Correlation between Serum Concentrations of Zinc and Copper with Serum Levels of SOD and GPX in Different Classes of Children with PEM

Groups		SOD*	GPx*
Marasmus	Zn	0.03	-0.14
	Cu	0.16	0.05
Kwashiorkor	Zn	-0.39	-0.54
	Cu	-0.39	-0.31
Marasmic-kwashiorkor	Zn	-0.31	-0.41
	Cu	-0.51	-0.48
Controls	Zn	0.12	-0.07
	Cu	0.07	-0.08

* These are R values. The coefficient of correlation (R) was carried out using the Pearson Correlation analysis. SOD = Superoxide dismutase, GPx = Glutathione peroxidase, Zn = Zinc, Cu = Copper.

CHAPTER FIVE

DISCUSSION

In this present study, the status of both antioxidant enzyme activities and some relevant trace elements and their possible relationship among under-five children with marasmus, kwashiorkor and marasmic-kwashiorkor as well as apparently healthy children were examined. Majority of the subjects in the study were Hausas and were from lower socioeconomic status which contributed to their inadequate nutritional intake as a result of poverty. Decreased activities of both SOD and GPx in protein-energy malnourished children with marasmus, kwashiorkor and marasmic-kwashiorkor may be due to deficiency of certain trace elements which forms the integral part of these enzymes (El Hassan *et al.*, 2004). Cu and Zn are an integral part of Cu-Zn SOD containing enzyme (Vallee and Auld 1990) and Se which is an integral part of Se-GPX containing enzyme (Levander and Burk 1990). It may also be due to harmful levels of free radicals found in children with protein-energy malnutrition (PEM) (Golden *et al.,* 1990). However, reduction of SOD and GPx activities in kwashiorkor and marasmic-kwashiorkor compared with marasmus, may be related to the severity of the disease. These results are in agreement with the report of El Hassan *et al.,* (2004) and Golden and Ramadath (1987).

Biochemical evidence of zinc and copper deficiencies has been reported in children with PEM (Sive *et al.,* 1993). Such evidence has also been provided by the present study, with reference to copper but not zinc. Increase in serum zinc levels in children with PEM may be due to environmental zinc contamination, or it could be possible that some of the PEM children were already receiving zinc supplement since PEM is associated with diarrhoea and

zinc supplementation has been found to reduce the duration and severity of diarrhoea (Bhutta *et al.,* 1999). In addition, oedema fluid has been found to contain substantial amount of zinc which is protein bound (Kumar and Rao, 1973). Although, there was no significant difference in serum zinc levels between marasmus, kwashiorkor and marasmic-kwashiorkor, but there was significant difference between children with PEM and controls. Zinc deficiency in apparently healthy children (controls) may be due to, dietary deficiency such as abnormally low zinc content in human milk or the low availability of zinc in cow's milk which is even lower than human milk (Casey *et al.,* 1981). Another contributing factor could be low content of zinc in the soil or phytate which is present in most foods of plant origin like whole grain cereals, which constitute the most important sources of nutrients for children in our environment, and may also inhibit zinc absorption (Dijkhuizen *et al.,* 2004). These results are in agreement with Ashour *et al.,* (1999), who reported elevated zinc levels in children with PEM compared with their controls, and also in agreement with Hegazi, (2002) who reported low serum zinc level in apparently healthy children in developing countries compared with the children of developed countries. The present work reveal a positive correlation between serum levels of zinc and copper with SOD activity in children with marasmus and controls, but show a negative correlation between serum levels of zinc and copper with SOD activity in children with kwashiorkor and marasmic-kwashiorkor. However, this study has not demonstrated any positive correlation between serum zinc levels and GPx activity in all the groups.

As regards to copper, low level of serum copper in marasmic children may be due to reduction in albumin or ceruloplasmin (copper-carrying protein in the blood) which is attributed to its excessive loss, destruction or inability to be synthesized leading to lack of

copper transport to the liver (Hansen and Lehman, 1969). Another contributing factor that may lead to copper deficiency in marasmic cases is repeated bouts of acute and chronic diarrhoea, as well as malabsorption which are common with PEM children (Mehta *et al.*, 1984). The results of the present study are in agreement with reports by Ibrahim (1990) and Ashour *et al.,* (1999). The present study also reveal a positive correlation between serum copper levels and GPx activity in children with marasmus, but show a negative correlation between serum copper levels and GPx activity in kwashiorkor, marasmic-kwashiorkor and control groups.

The low serum total protein and albumin levels in children with PEM may reflect liver synthetic failure or diseases such as chronic hepatitis among the PEM children. However, decrease in levels of total protein and albumin in children with kwashiorkor and marasmic-kwashiorkor compared with marasmus suggests an abnormally low protein in the blood (hypoproteinemia), due to severe limitation of protein intake in the diet. Decreased serum protein decreases the osmotic pressure of the blood, leading to loss of fluid from the intravascular compartment or the blood vessels to the interstitial tissues, resulting in oedema. The present results are in agreement with reports by Ashour *et al.,* (1999) and Ugwuja *et al.,* (2007).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The major findings of this work can be summarized as follows:

i. The serum total protein, albumin, SOD and GPx were significantly lower ($p<0.05$) in PEM children than in controls.

ii. Serum concentration of zinc was significantly higher ($p<0.05$) in all the PEM children than in the controls.

iii. Serum concentrations of copper were lower in marasmus and marasmic-kwashiorkor than in controls, but significantly higher ($p<0.05$) in kwashiorkor compared with that of the marasmic-kwashiorkor.

iv. There were correlations between these serum levels of trace elements (Zn, Cu) and antioxidant enzymes (SOD, GPx) in children with PEM and controls. Marasmus (SOD-Zn: 0.03, SOD-Cu: 0.16, GPx-Zn: -0.14, GPx-Cu: 0.05), kwashiorkor (SOD-Zn: -0.39, SOD-Cu: -0.39, GPx-Zn: -0.54, GPx-Cu: -0.31), marasmic-kwashiorkor (SOD-Zn: -0.31, SOD-Cu: -0.51, GPx-Zn: -0.41, GPx-Cu: -0.48) and control (SOD-Zn: 0.12, SOD-Cu: 0.07, GPx-Zn: -0.07, GPx-Cu: -0.08).

6.2 Conclusion

Antioxidants play a very important role in the body defense system against reactive oxygen species. The results of this study show decreased SOD and GPx activities in children with PEM which make them liable to certain oxidative effect of the reactive oxygen species. The children

with PEM are also characterized by decreased serum copper, total protein and albumin, all pointing to the fact that children with PEM are predisposed to high oxidative stress.

6.3 Recommendations

1. Routine laboratory investigation of antioxidants should be done for effective management of PEM.
2. All under-five children attending the out-patient clinics should be thoroughly examined for signs of malnutrition especially in developing countries.
3. Promotion of good hygienic practice, health education, immunization programmes and family planning in Nigeria should be intensified.
4. Exclusive breast feeding for six months, adequate complementary feeding from six months and food fortification should also be encouraged among under-five children in Nigeria as measures against developing PEM.
5. Further studies should be done on zinc levels among apparently healthy and malnourished children in Nigeria.

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APPENDIX I

INFORMED CONSENT FORM FOR PROTEIN-ENERGY MALNOURISHED CHILDREN ATTENDING AHMADU BELLO UNIVERSITY TEACHING HOSPITAL, ZARIA

Name of principal investigator:

Name of organization:

Title of Research:

Phone Number:

Introduction: I am Njoku Stella Ujunwa of Department of Biochemistry, Ahmadu Bello University, Zaria. I am doing a research on Protein-Energy Malnutrition, a disease which is very common in our country. I am going to give you information and invite you to be part of the research. Before you decide, you can talk to anyone you feel comfortable with about the research. If there is anything you do not understand, please feel free to ask questions at any point in time.

Purpose of research: Protein-Energy Malnutrition (PEM) is a common and dangerous disease among under-five children in this region. Although opinion on the relationship between trace elements and PEM is known, there is no sufficient evidence or data on the relationship between trace elements and antioxidant enzymes among under-five children with PEM. The reason we are doing this research is to broaden the understanding of the relationship between trace elements and antioxidant enzymes in protein-energy malnourished children in Nigeria with a view to formulating nutrition intervention programs and policies targeted at reducing the prevalence of this disease and its detrimental effect among under-five children.

Type of Research intervention: The research would involve the collection of 2ml of blood from the arms. Your child may feel a slight pain in the process, but it will not cause any problem to his/her health. The blood would be collected only once throughout the research period of about six months.

Participant selection: I am inviting Protein-Energy Malnourished children between the ages of 1 –59 months that are in stable condition and attend ABUTH to participate in the research so as to help in reducing the prevalence of this disease and its detrimental effect among under-five children.

Voluntary Participation: It is entirely your choice whether to participate or not in the research as all services you receive in this clinic would continue and nothing will change.

Confidentiality: I would not be sharing the identity of those participating in the research. However, the knowledge that we get from this research may be published in order that other interested people may learn from our research.

APPENDIX II

CERTIFICATE OF CONSENT

I have read the earlier information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

Print Name of Participant _____

Signature of Participant _____

Date _____

Day/month/year

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness _____ AND Thumb print of participant

Signature of witness _____

Date _____

Day/month/year

Statement by the researcher

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. Two milliliter of blood would be collected from the participant.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability.

I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this Informed Consent Form has been provided to the participant.

Print Name of Researcher ----------

Signature of Researcher ----------

Date ----------

Day/month/year

APPENDIX III

QUESTIONNAIRE FOR THE STUDY ON RELATIONSHIP BETWEEN SOME TRACE ELEMENTS AND ANTIOXIDANT ENZYMES AMONG UNDER-FIVE CHILDREN WITH PROTEIN-ENERGY MALNUTRITION.

Bio data of respondents

Research ID _____ Gender_____ Age: _____

Date of birth: _____ State of origin _____

Ethnic background: ___ _____ Mother's tribe _____

Father's tribe _____

Socioeconomic background

Father's occupation: _____ Mother's occupation _____

Father's level of education _____ Mother's level of education _____

For Female respondents:

How many children do you have? _____

What is the Birth order? _____

Any family health history? _____ (specify)

Was the child exclusively breast-fed? _____

When did you stop breast feeding? _____

What type of food does the child eat? _____

How many times do you feed the child in a day? _____

What do you usually use to feed the child? _____

(Spoon feeding/Bottle feeding)

How many times does the child pass stool in a day? _____

What is the nature of the stool? _____

(Watery/Thick)

How often does the child fall ill in a month? _____

How often do you visit the hospital in a month? _____

Has the child been receiving any food supplement? _____

APPENDIX IV

SOCIO-ECONOMIC SCORE

The classification of Olusanya *et al.,* (1985) for socio-economic scores based on father's occupation and mother's education:

A. Husband (father's occupation)

Score: 1. Professionals, top civil servants, politicians and businessmen.

2. Middle-level bureaucrats, technicians, skilled-artisans and well to do traders.

3. Unskilled workers and those in general whose income would be at or below the national minimum wage.

B. Mother's education level

Score: 0 Education up to University level

1. Secondary or tertiary level below the University

2. No formal education or up to primary level only

The socio-economic class of the child's parents was obtained by adding the scores from A and B above and graded I-V as appropriate, for example:

Score (1) from A plus score (0) from B = social class I

Score (2) from A plus score (0) from B = social class II

Score (2) from A plus score (1) from B = social class III

Score (2) from A plus score (2) from B = social class IV

Score (3) from A plus score (0) from B = social class III

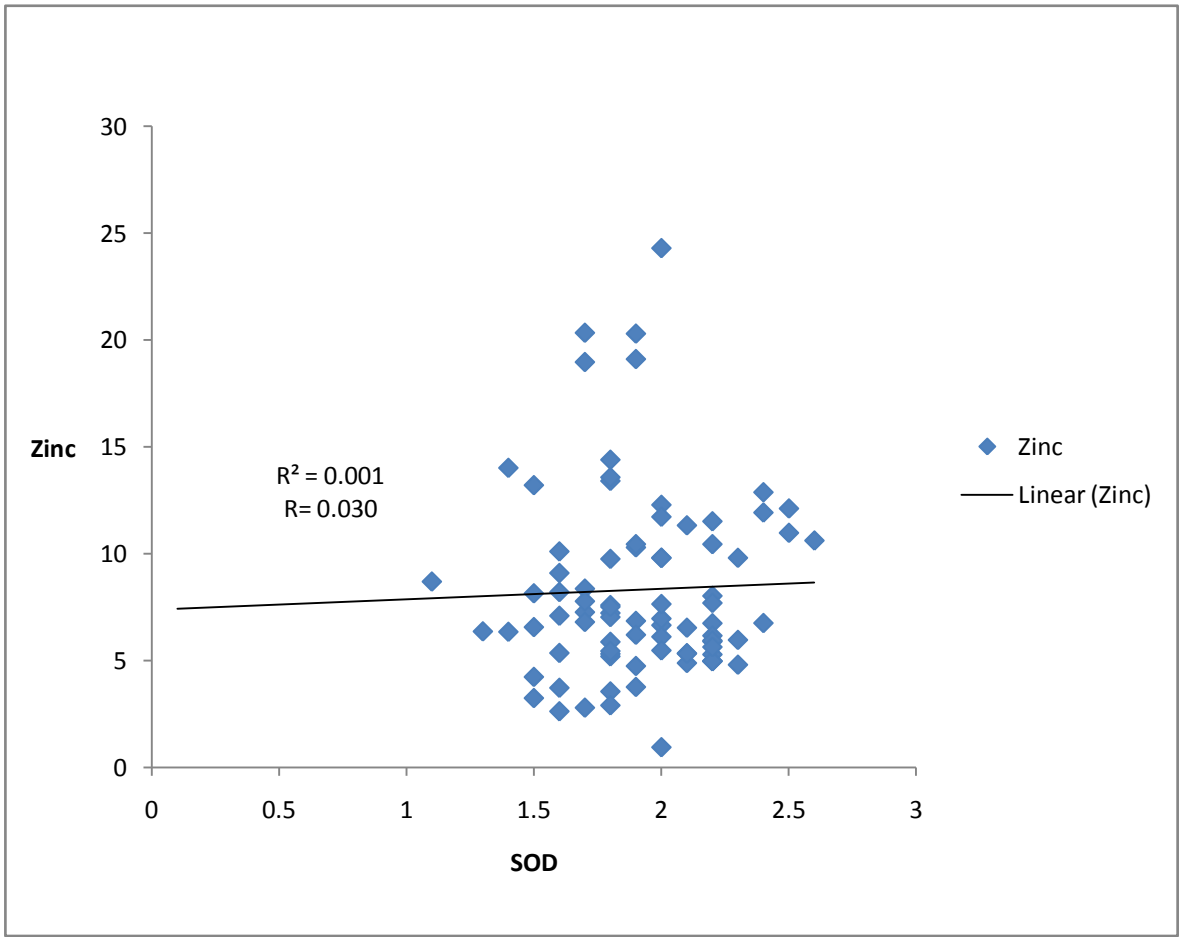
Score (3) from A plus score (1) from B = social class IV

Score (3) from A plus score (2) from B = social class V

Reference: Olusanya O, Okpere E, Ezimokhai M. The importance of socio-economic class in voluntary fertility control in a developing country. WAJM 1985;4:205-262

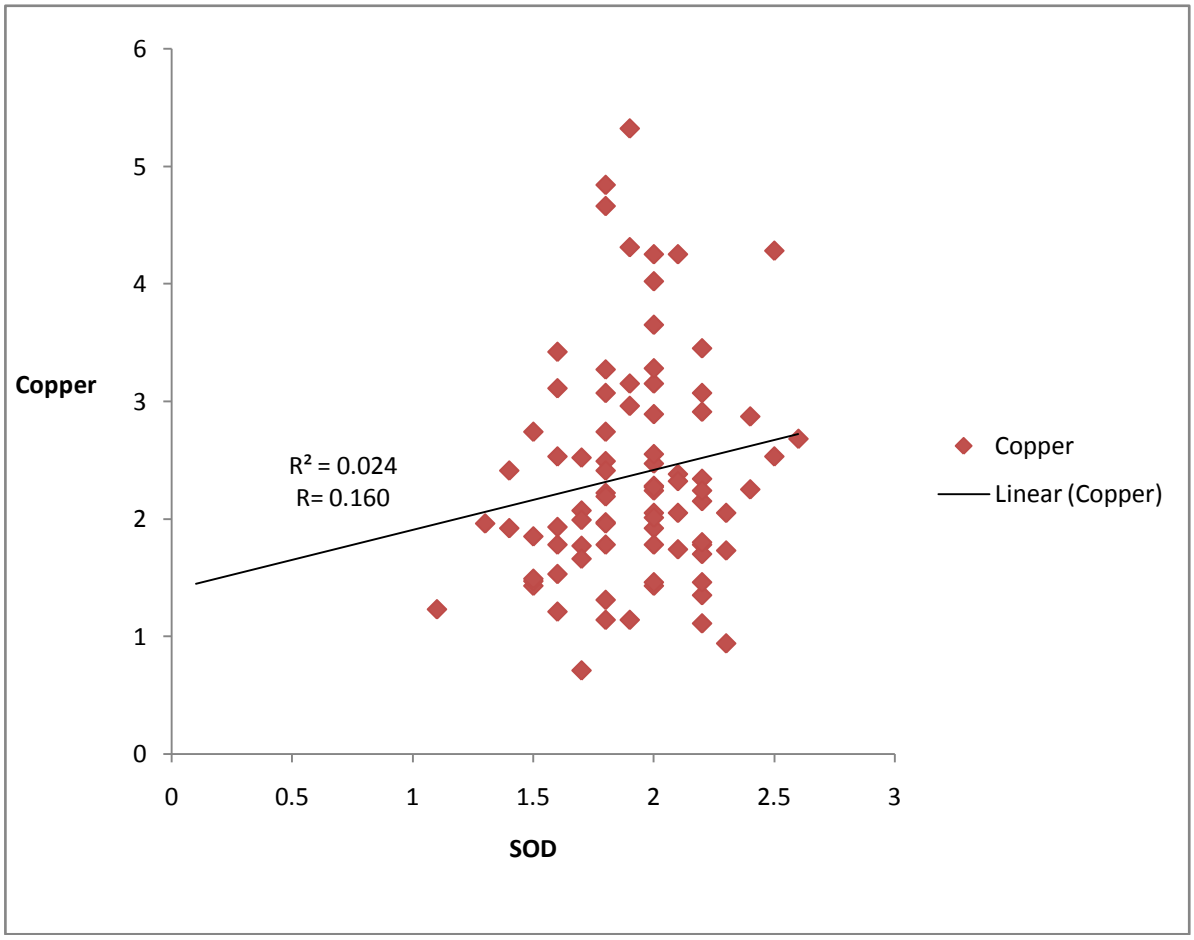
APPENDIX V

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF SOD IN MARASMUS



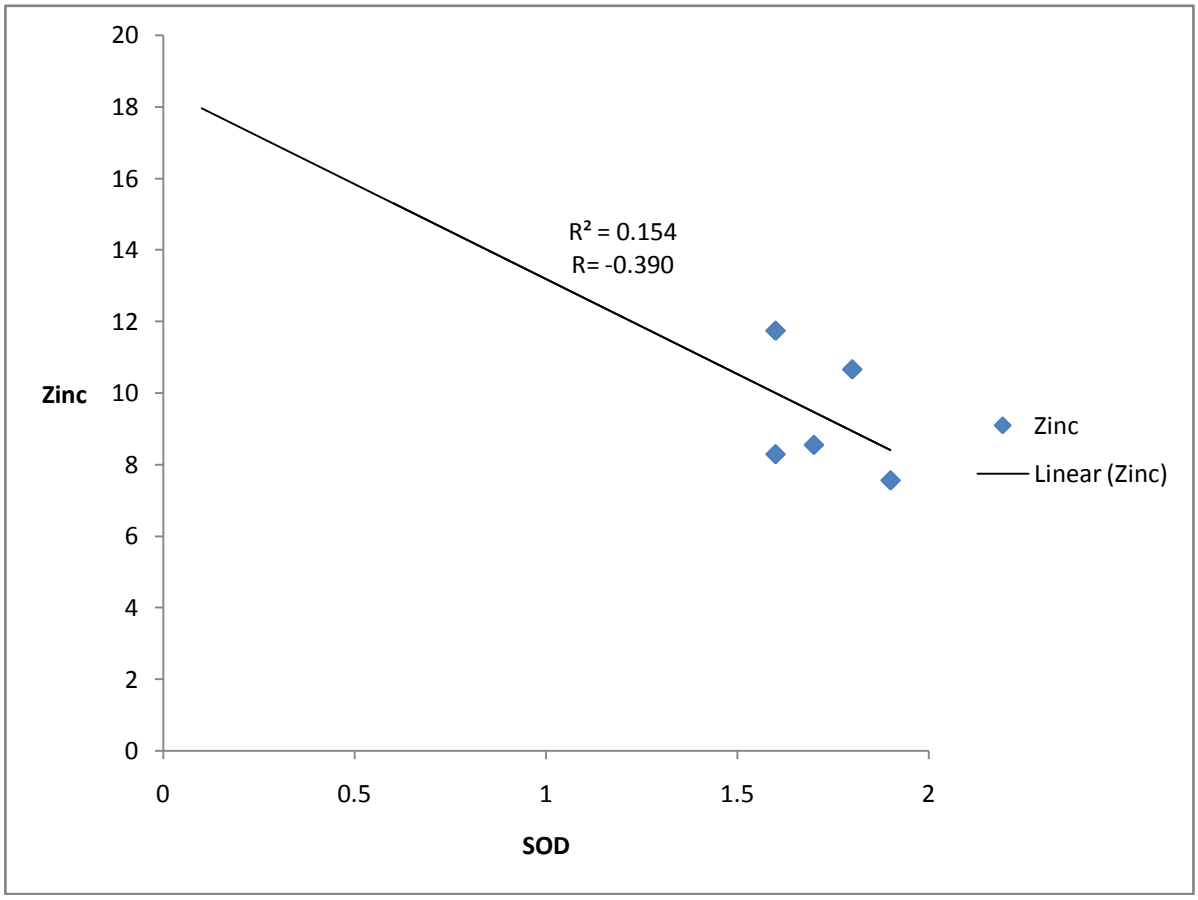
APPENDIX VI

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF SOD IN MARASMUS



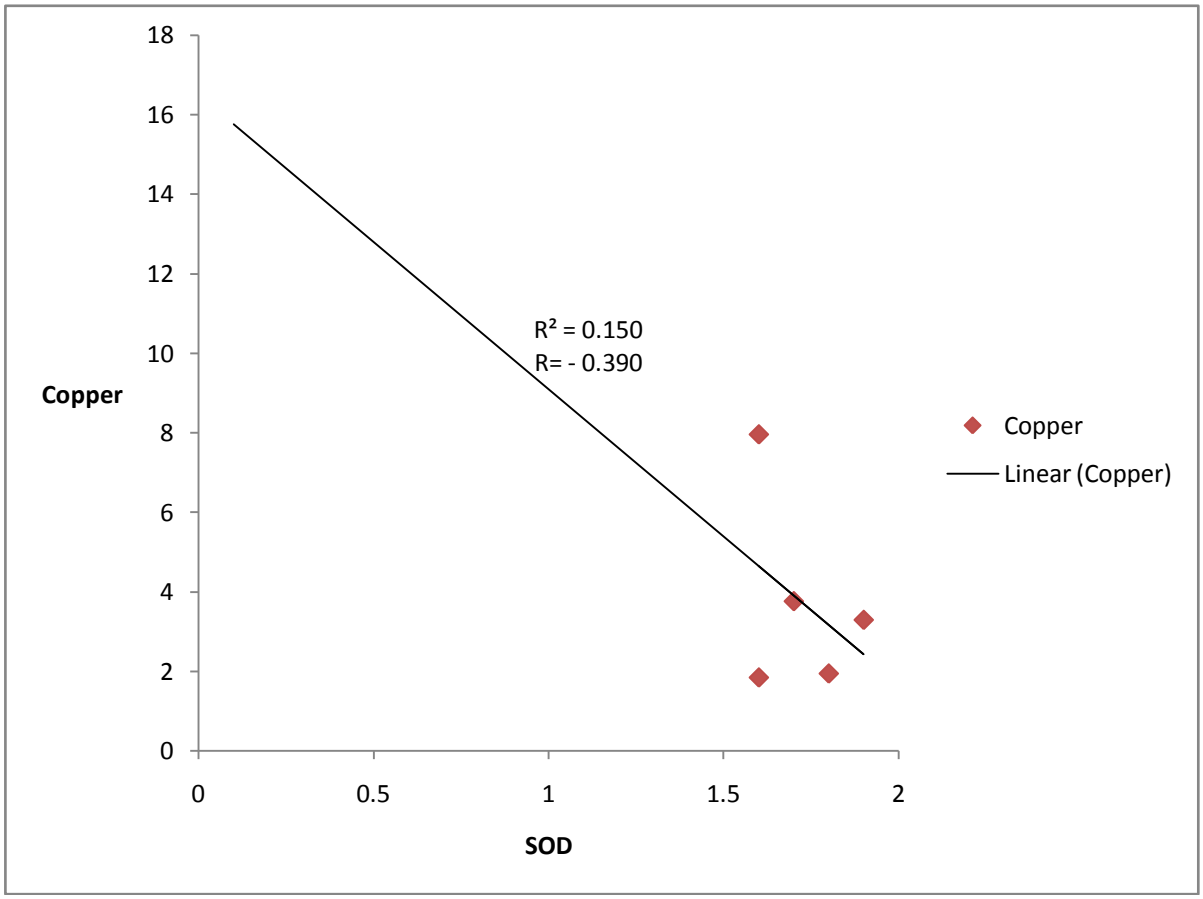
APPENDIX VII

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF SOD IN KWASHIOKOR



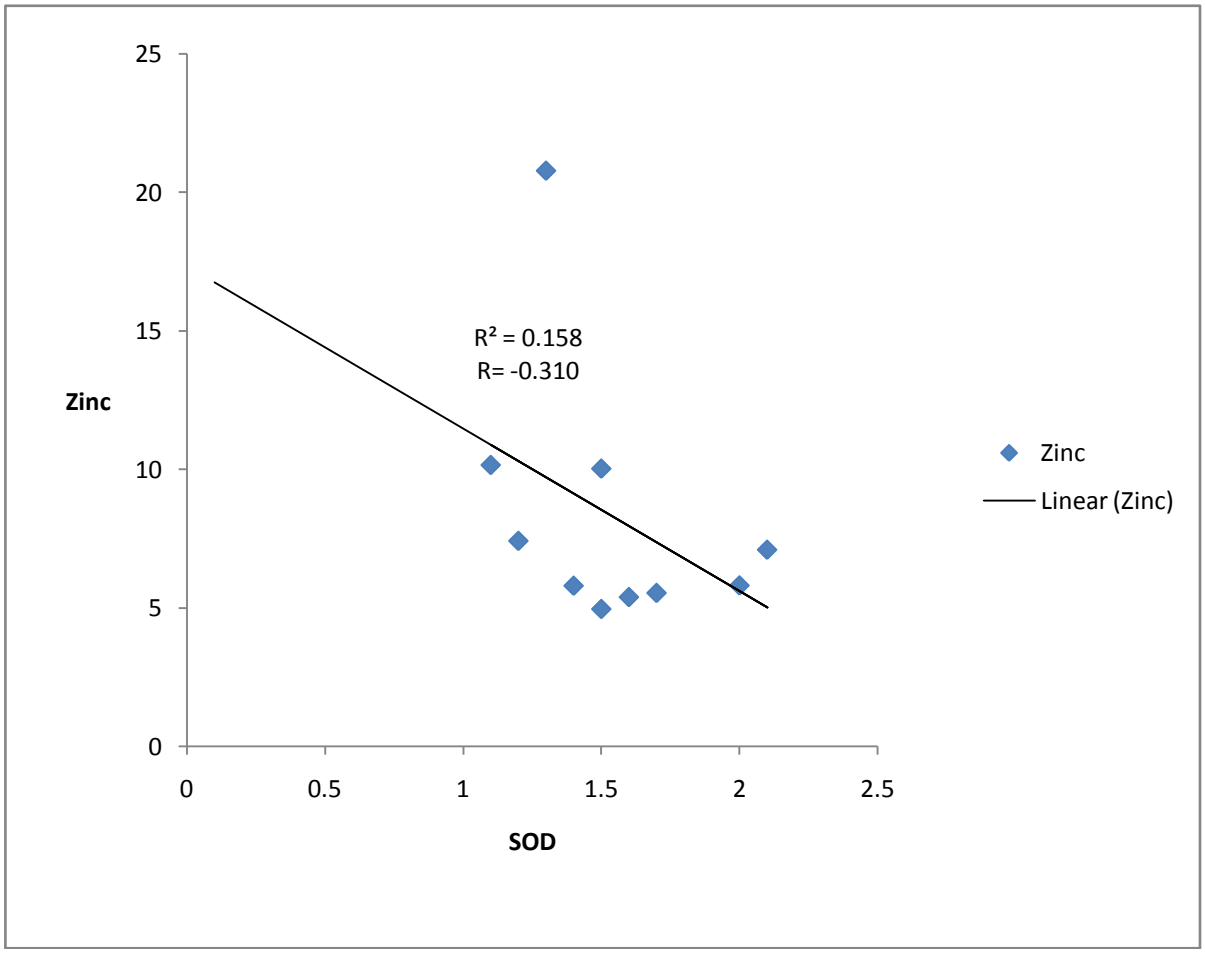
APPENDIX VIII

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF SOD IN KWASHIOKOR



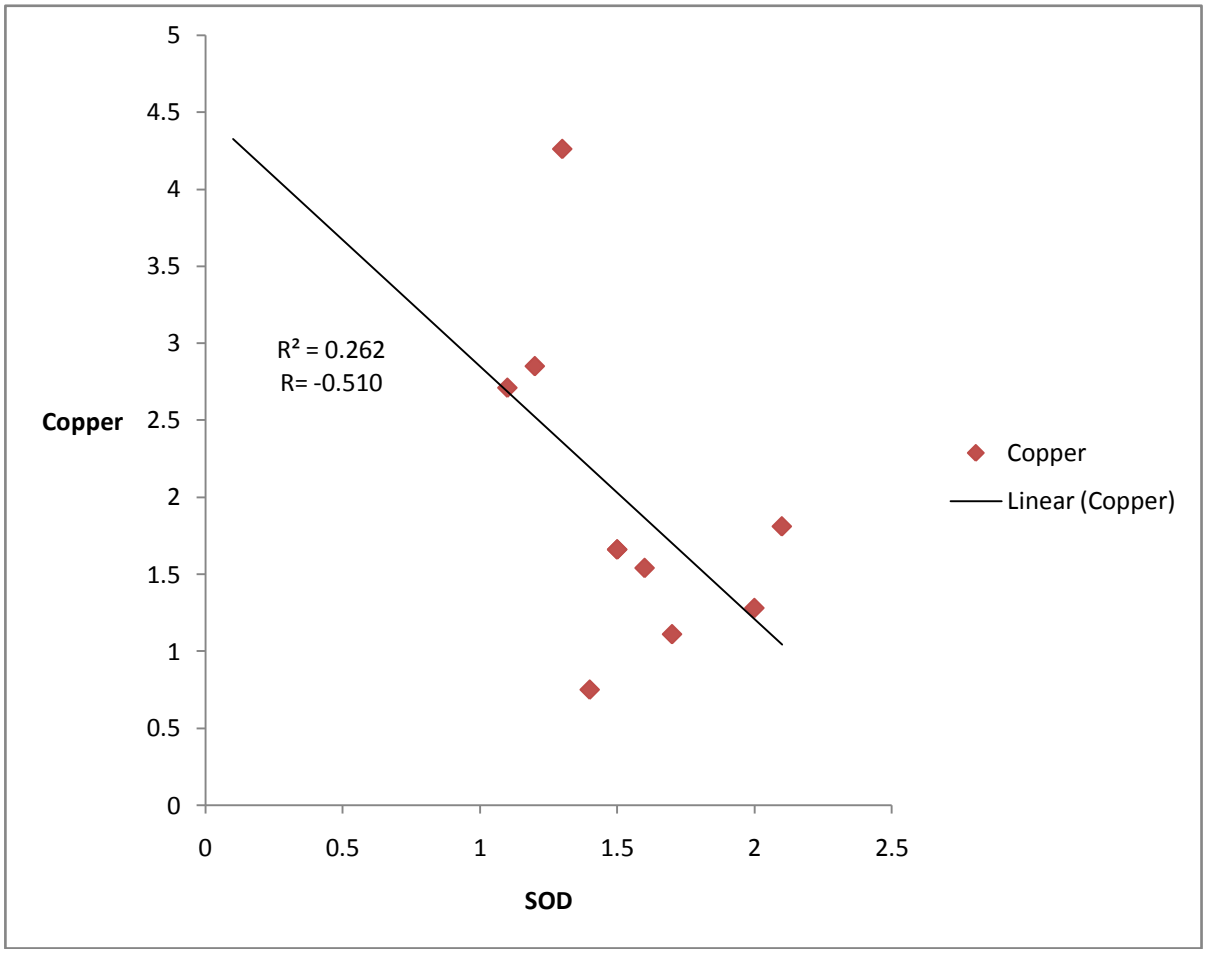
APPENDIX IX

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF SOD IN MARASMIC-KWASHIOKOR



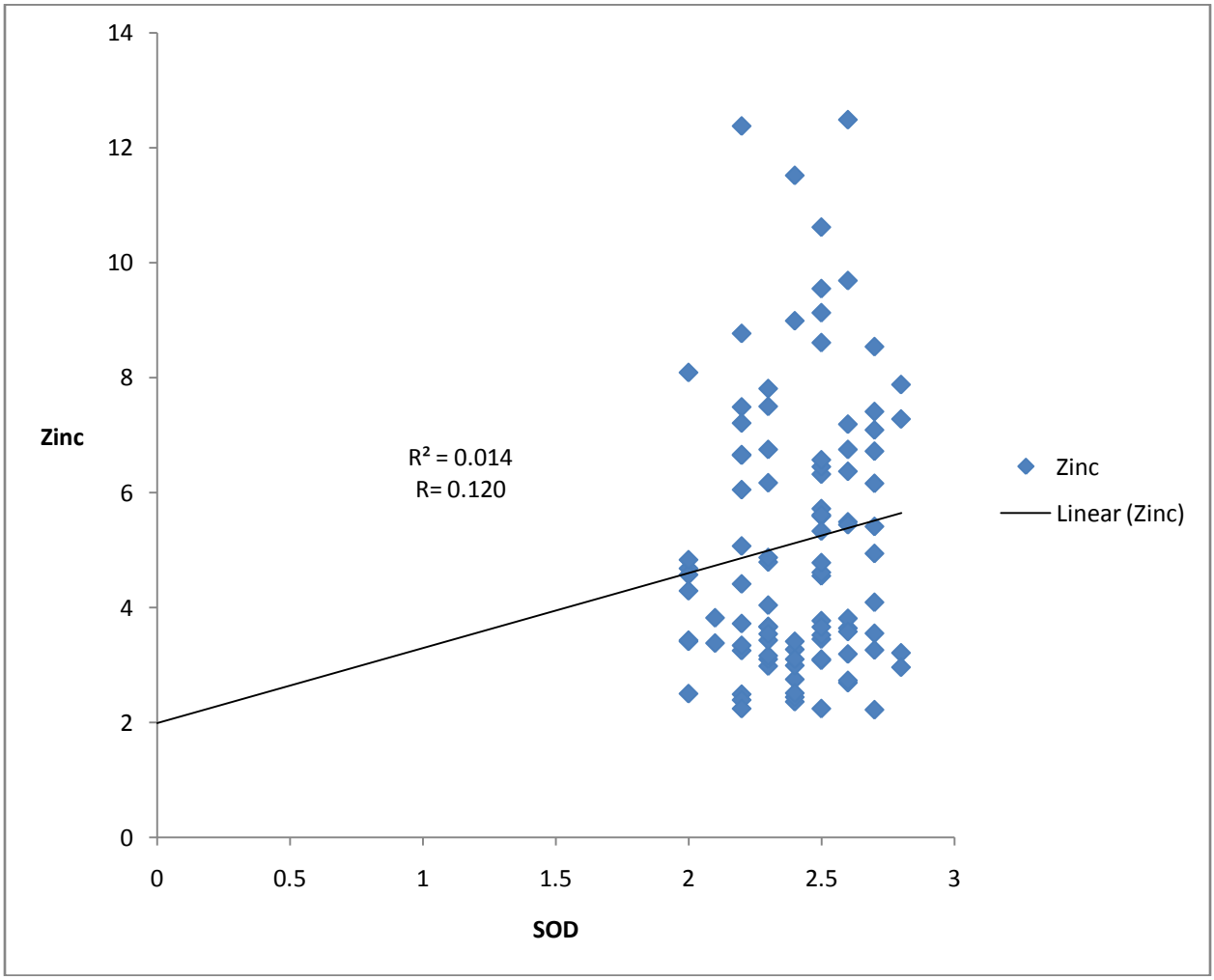
APPENDIX X

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF SOD IN MARASMIC-KWASHIOKOR



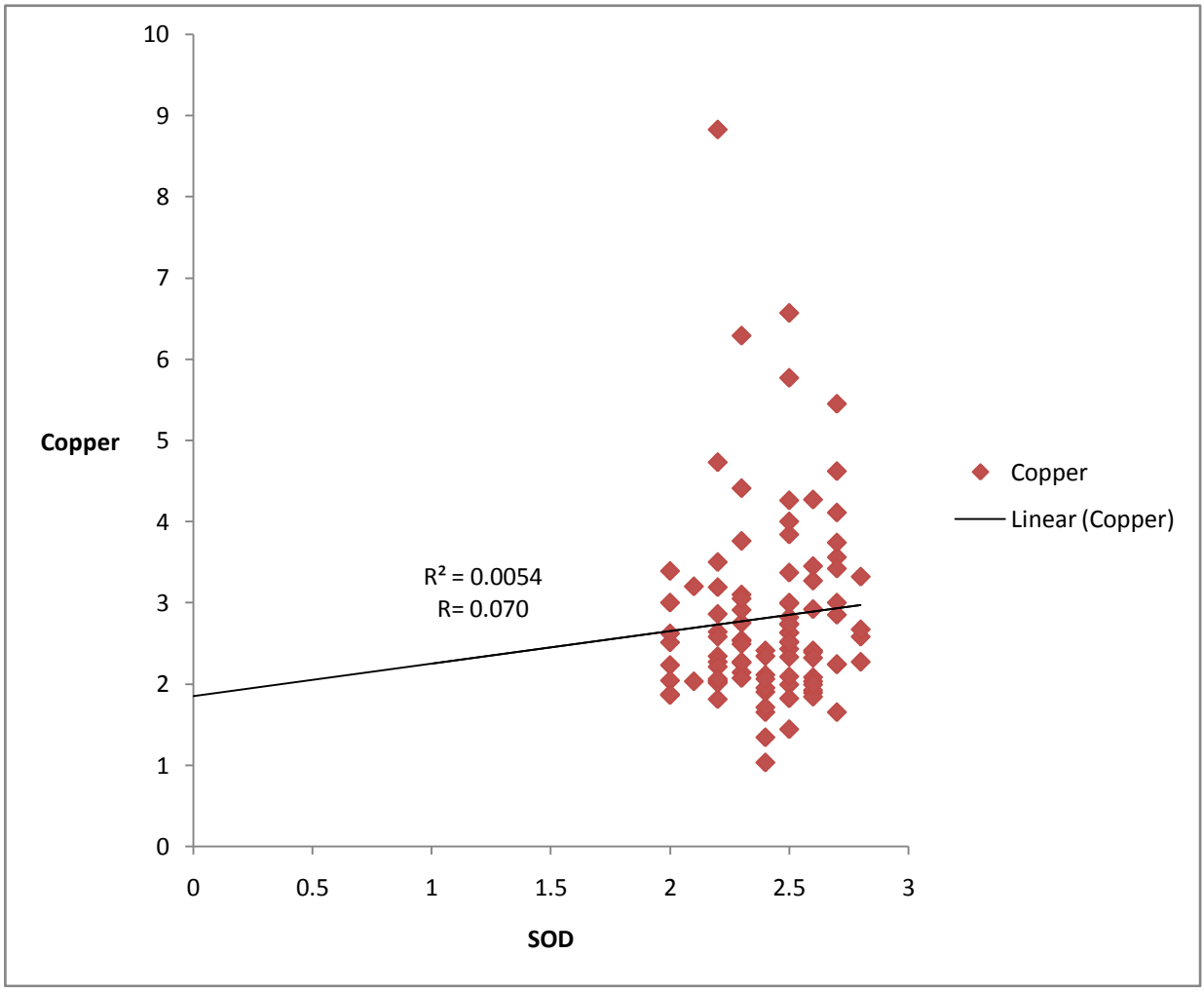
APPENDIX XI

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF SOD IN CONTROL



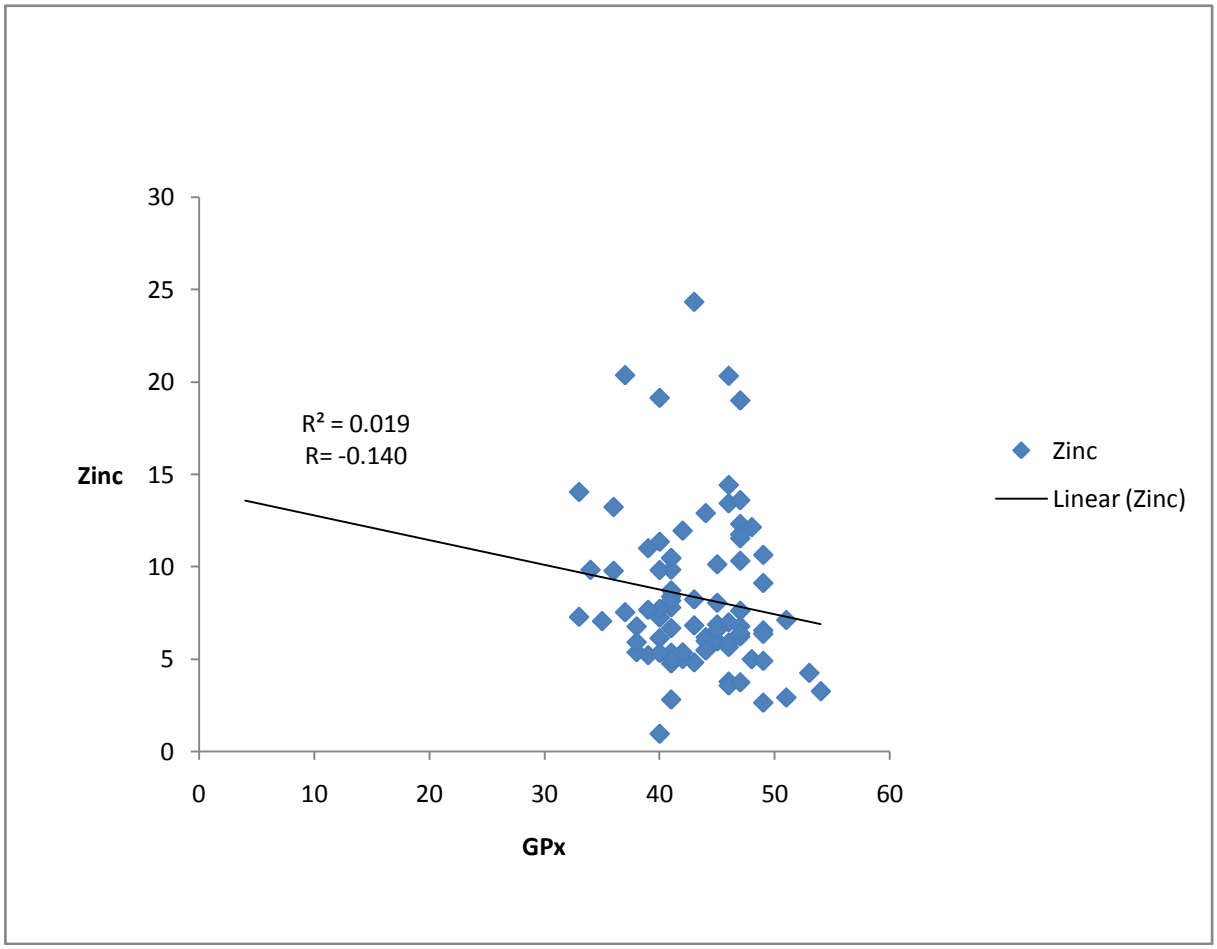
APPENDIX XII

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF SOD IN CONTROL



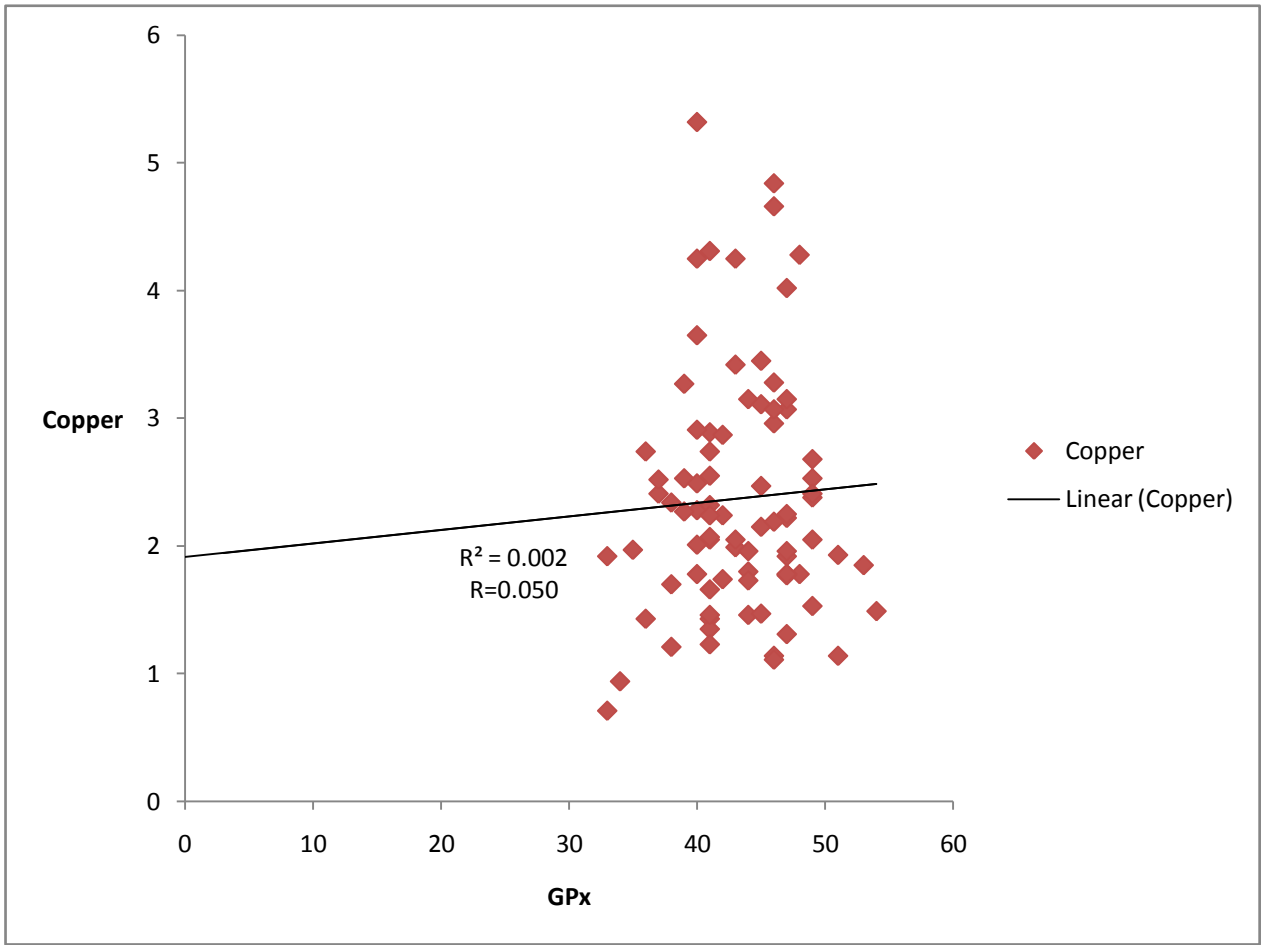
APPENDIX XIII

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF GPx IN MARASMUS



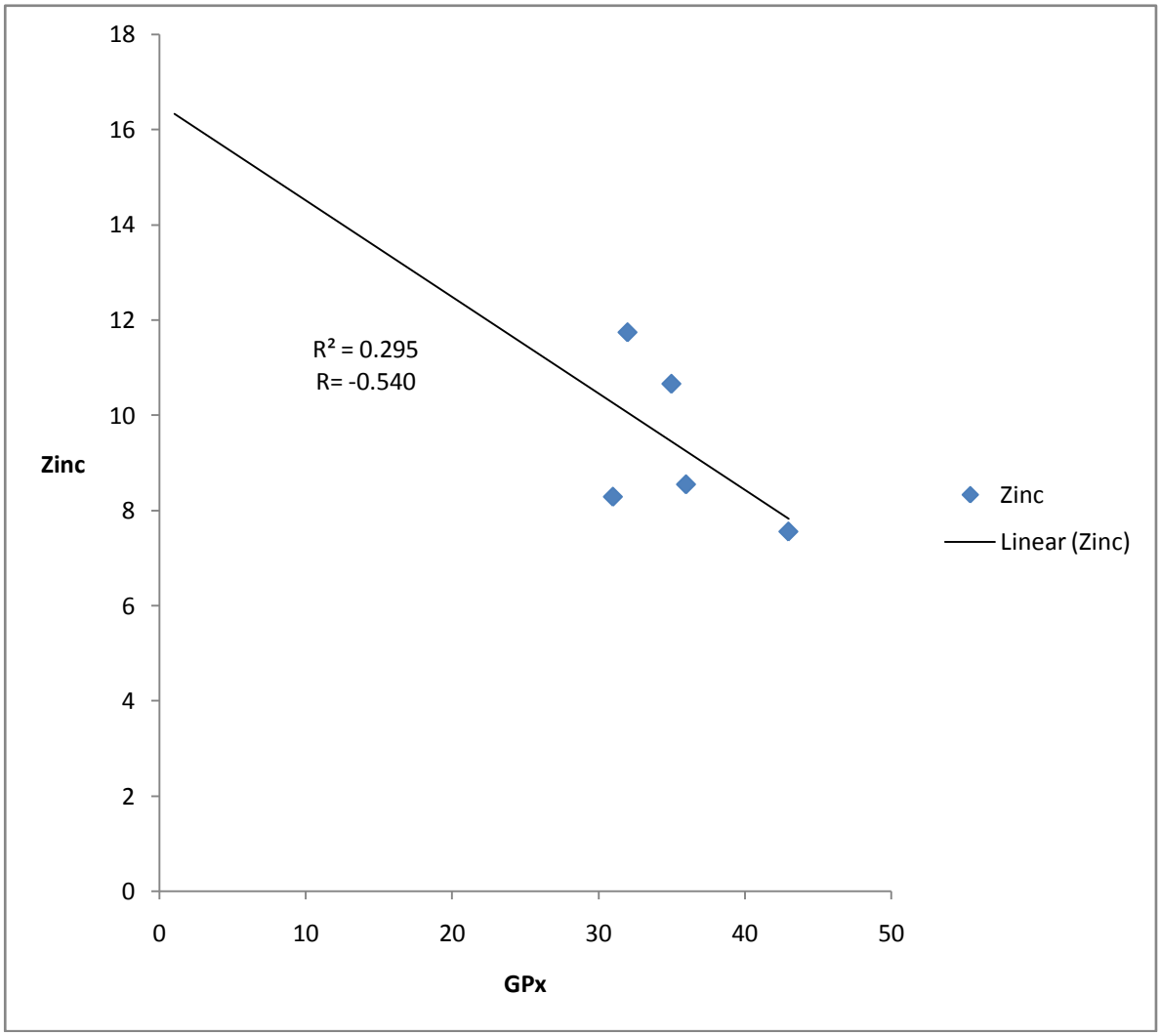
APPENDIX XIV

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF GPx IN MARASMUS



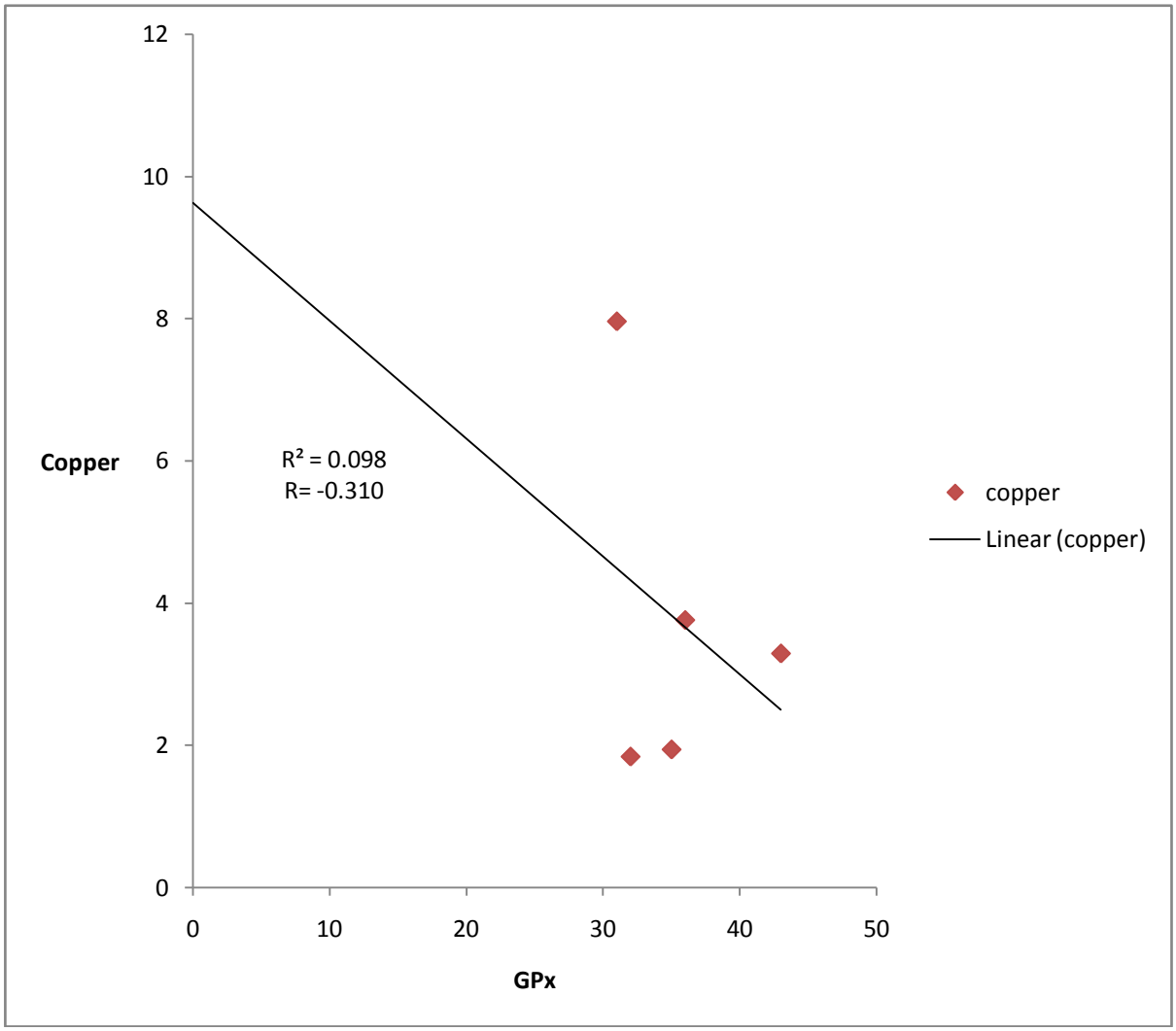
APPENDIX XV

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF GPx IN KWASHIORKOR



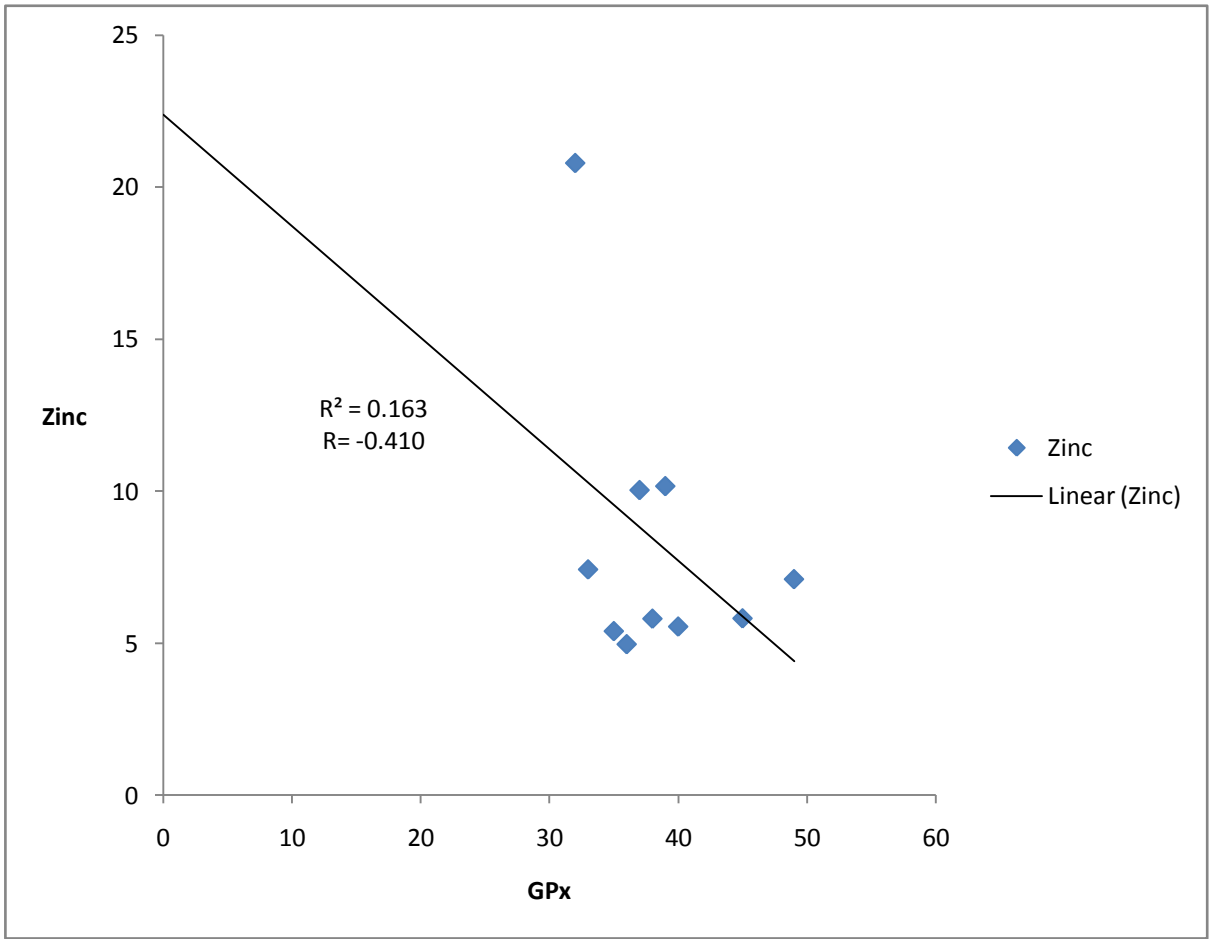
APPENDIX XVI

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF GPx IN KWASHIORKOR



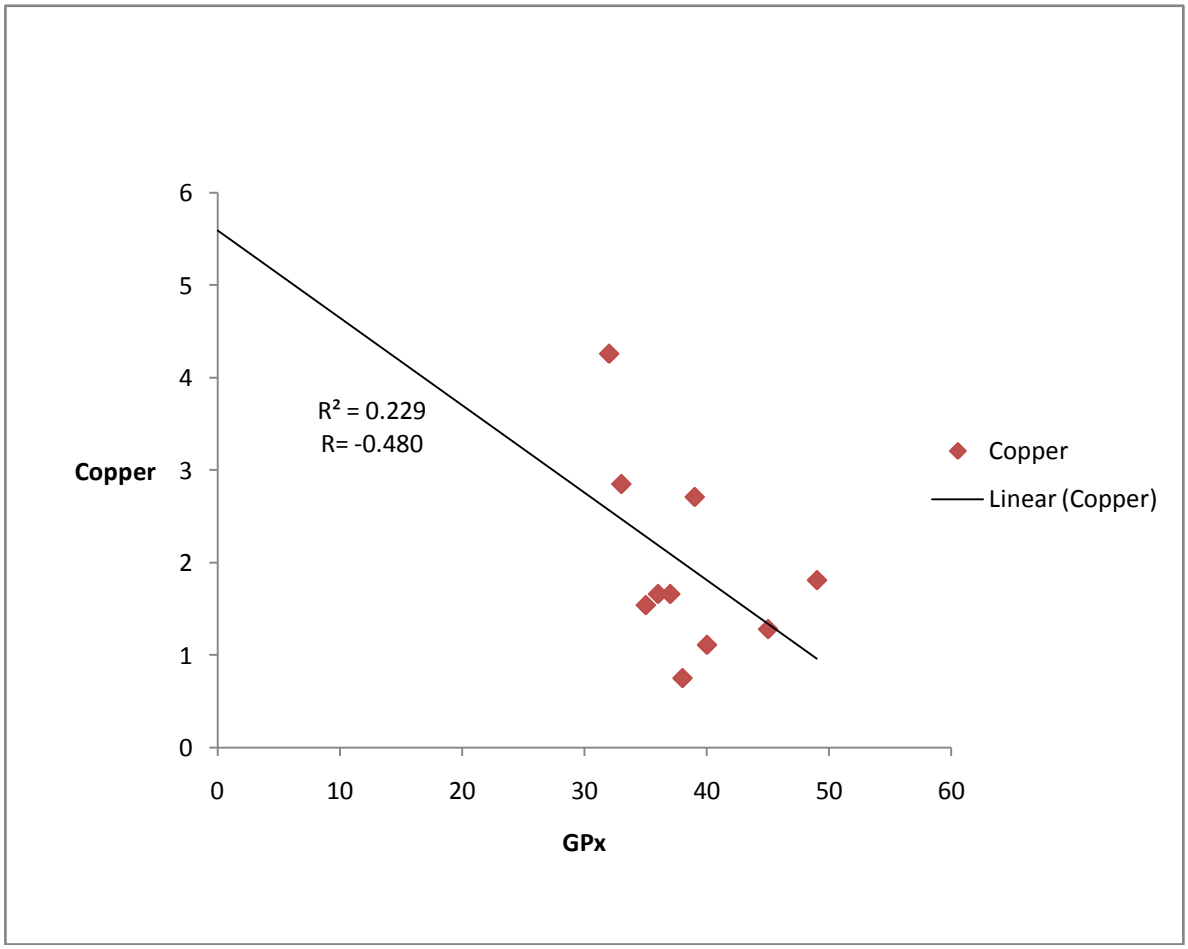
APPENDIX XVII

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF GPx IN MARASMIC-KWASHIORKOR



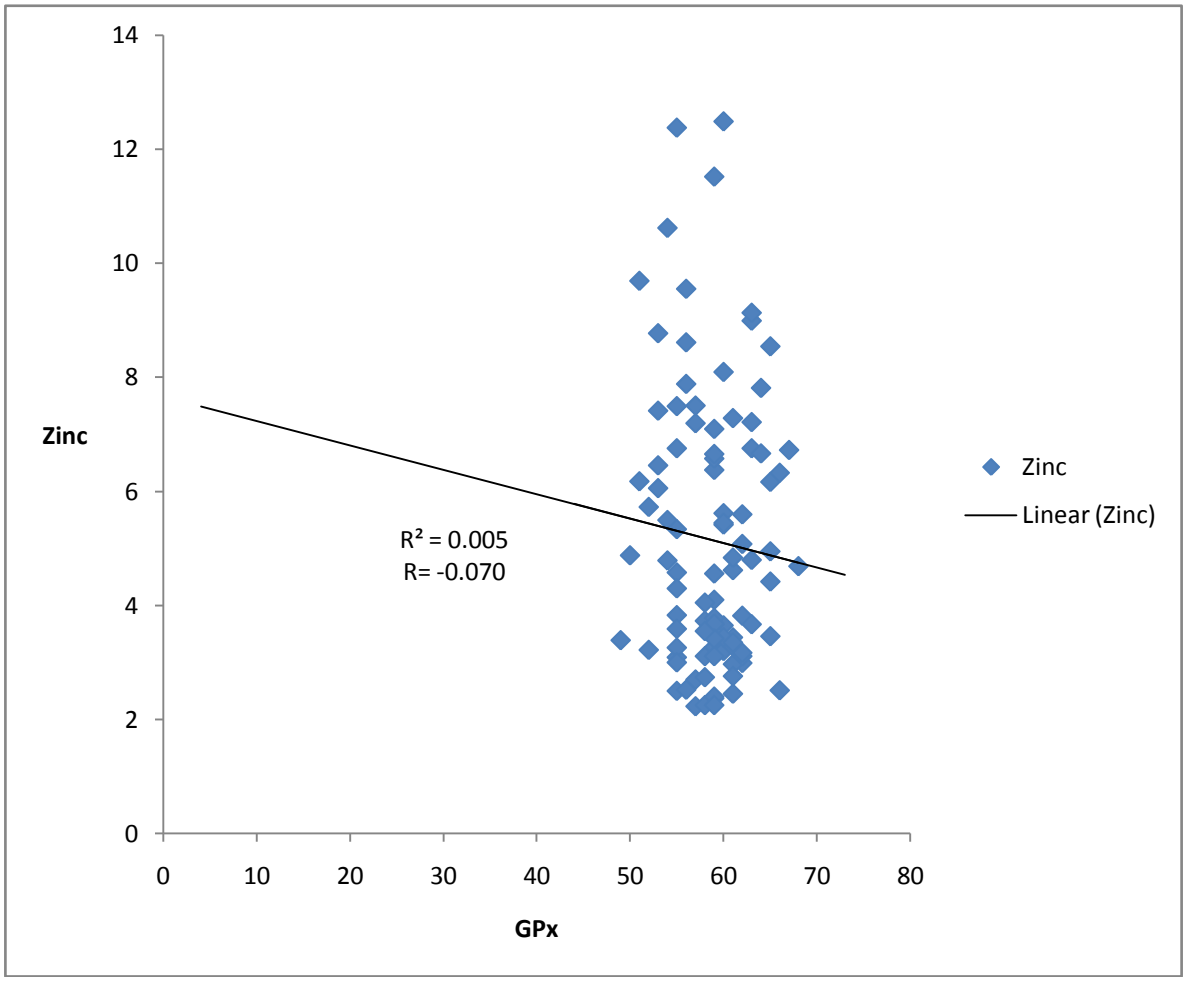
APPENDIX XVIII

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF GPx IN MARASMIC-KWASHIORKOR



APPENDIX XIX

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF ZINC WITH SERUM LEVELS OF GPx IN CONTROL



APPENDIX XX

PEARSON CORRELATION BETWEEN SERUM CONCENTRATIONS OF COPPER WITH SERUM LEVELS OF GPx IN CONTROL

