Investigation of Serum Markers of Oxidative Stress among Breast Cancer Patients in Tikur Anbessa Specialized Teaching Hospital, Addis Ababa, Ethiopia

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Declaration sheet

This is to certify that this master's thesis entitled "***Investigation of Serum Markers of Oxidative Stress among Breast Cancer Patients in Tikur Anbessa Specialized Teaching Hospital, Addis Ababa, Ethiopia"***, was prepared by Yimenashu Mamo kassaye, and Submitted to the department of Biochemistry in requirements for partial fulfillment of degree of Master's science in Medical Biochemistry, with regulation of university and meet acceptance standards with respect to originality and quality.

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Student declaration Sheet

I, the undersigned, declare that this research paper titled on Investigation of Serum Markers of Oxidative Stress among Breast Cancer Patients in Tikur Anbessa Specialized Teaching Hospital, Addis Ababa, Ethiopia is my original work and has not presented for a degree in any other university, and that all sources of materials used for the research have been properly and suitable acknowledged.

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Abstract

Background: Breast cancer has become a major public health problem in developing regions, as incidence rate is particularly growing in these regions of the world. Oxidative damage and modification of biomolecules including proteins, lipids and DNA can increase the risk of mutagenesis. It has also been suggested to play an important role in breast carcinogenesis, and progression of the disease.

Objective: The aim of this study was to investigate the serum markers of oxidative stress among breast cancer patients in Tikur Anbesa specialized teaching hospital, Addis Ababa, Ethiopia.

Methods: The comparative cross sectional study was conducted on a total of 119 study participants (95 breast cancer patients and 24 female controls without malignancy). Breast cancer patients were classified based on their clinical stages at diagnosis (stage I, II, III and IV).The parameters measured were serum levels of malondialdehyde (MDA), protein carbonyl, reduced glutathione (GSH) , total antioxidant capacity (TAC), uric acid, and albumin using their standard methods. Data analyses were done with SPSS version 21 system.

Results: The present study result demonstrated that there were statistically significantly higher serum MDA and protein carbonyl levels in breast cancer group than in control group while the levels of TAC, GSH, albumin and uric acid were decreased (p-value < 0.05). Patients with the highest mean serum MDA and median protein carbonyl levels were those with stages III and IV as compared stages I and II (p-value < 0.05). Among the case groups, as breast cancer progresses to a higher stages, the levels of MDA, protein carbonyl, TAC and albumin were found to increase ($p < 0.05$). We also found significant decrease in the mean levels of GSH in stage IV as compared to other stage ($p < 0.05$). Moreover, a negative correlation was observed between serum levels of GSH versus serum levels MDA and protein carbonyl.

Conclusion: Breast cancer patients suffer a high degree of oxidative stress which is associated with decrease in TAC and GSH levels as well as significant degree of lipid peroxidation and protein oxidation. Clinical stages of breast cancer were associated with oxidative stress. The oxidative and antioxidant markers showed a negative correlation in patients with breast cancer.

Key words: Breast cancer, serum marker, oxidative stress

List of Acronyms and abbreviation

4-HNE	4-hydroxy-2-noneal
8-OHdG	8-hydroxy-2'-deoxyguanosine
BRCA	Breast cancer susceptibility gene
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
GPx	Glutathione peroxidase
GSH	Reduced Glutathione
GSSG	Glutathione disulphide
H_2O_2	Hydrogen peroxide
IGF	Insulin-like growth factor
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7
MDA	Malondialdehyde
PTEN	Phosphatase and tensin homolog
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TNM	Tumour-node-metastases
Trx	Thioredoxin

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1 . Introduction

1.1 Background

Breast cancer refers to cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. It is a clonal disease; a single transformed cell- the end results a series of somatic (acquired) or germ line mutations is able to express full malignant potential (Braunwald *et al.,* 2001). Breast cancer typically produces no symptoms when the tumour is small and most treatable. It is therefore important for women to follow the recommended screening guidelines for detection of breast cancer at an early stage before the symptoms develop. When it has grown to a size that can be felt, the most common physical sign is a painless mass (Braunwald *et al.,* 2001).

Breast cancer is the most common cancer in women both in the developed and less developed world. It is estimated that worldwide over 508,000 women died in 2011 due to breast cancer (WHO, 2013). Breast cancer was the most commonly diagnosed cancer and the second leading cause of cancer death among women in 2008 in Africa (Jemal *et al.*, 2012). Incidence rate of breast cancer increases quickly until menopause. Incidence increases at slower rate or can even decreases slightly (low–incidence countries) after menopause (Hulka *et al.,* 2008). In developed countries, breast cancer incidence peak are between 55 and 60 years and between 45 and 50 years in developing countries (Leong *et al.,* 2010).

Currently, breast cancer has become a major public health problem in developing regions, as incidence rate is particularly growing in these regions of the world. Deaths of women from breast cancer during their most productive years could result in tragedy for families, food insecurity, and children withdrawal from school, increased work burden on children and loss of assets (Anderson *et al.*, 2008).

The incidence rate of breast cancer is highly correlated with "western lifestyle" since the cases are higher in developed countries than the developing countries (Ferlay *et al.,* 2010). In terms of mortality and incidence among women, sub-Saharan Africa is the only region where cervical cancer is equivalent to breast cancer which accounts approximately quarter of the total burden and is the most common cause of cancer death (23.2% of the total) in women (Stewart and Wild, 2014).

In Ethiopia, the predominantly rural environment (*e.g*., less processed food) and non-Westernized lifestyle (*e.g*., underweight, high physical activity, high number of children, and long period of breastfeeding, less hormonal contraceptives or replacement therapy) may reflect differences from other African countries. Also, the lack of awareness, low economic status, lack of knowledge, etc, may result in less treatment uptake (Bogale *et al.,* 2011).

In another study conducted in Tikur Anbesa specialized teaching hospital, clinically the majority of breast cancer cases had stage III disease. Invasive ductal carcinoma was the most frequent type. Over 80 % patients underwent modified radical mastectomy. During a short follow up approximately 50 % of patients were seen with recurrences. Only 4 cases were seen at 5 or more years (Ersumo, 2006). In Ethiopia, an estimated age-standardized incidence rate of 19.5 per 100,000 annually and an estimated age-standardized death rate of 11.8 per 100,000 females are reported. Women with breast cancer account for 19% of the total cancer patients (Kantelhardt *et al.*, 2014).

Risk factors of breast cancer

Risk factors of breast cancer depends on woman's cumulative lifetime exposure to endogenous estrogen and her periodic use of exogenous hormones including young age at menarche, delayed menopause, late first pregnancy, nulliparity and use of hormone replacement therapy or oral contraceptives increase the risk of breast cancer. On the other hand, late menarche, early menopause, early first pregnancy, high parity and prolonged lactation seem to have protective effect (Reeves *et al., 2009*; Newcomb *et al.,* 2011). Breast cancer risk have also shown to have correlation with increased density of the breasts as seen in mammography and benign breast lesions, especially atypical hyperplasias, (Tice *et al.,* 2013).

Breast cancer risk increases gradually with the more affected first-degree relatives a woman has. It has been estimated that approximately 7% of all breast cancers are hereditary (Hulka and Moorman 2001). Hereditary breast cancers usually emerge among younger women with mutation in one of the high-penetrance breast cancer susceptibility genes such as BRCA1, BRCA2, p53, PTEN and ATM. Mutations in *BRCA1* and *BRCA2* alone are responsible for roughly 80 to 90% of all hereditary breast cancers and about 5 to 10% of all breast cancer cases (De Jong *et al.,* 2002). Multiple exposures of therapeutic radiation to the chest for cancer at an early age (less than 20 years old) pose a high risk of developing breast cancer. Patients with Hodgkin's disease receiving radiotherapy at high doses are at high risk to develop breast cancer (Stovall *et al.,* 2008).

Low doses of alcohol consumption (*i.e*., ≤1 drink/day) increase the risk of breast cancer by about 4 % (Hamajima *et al,* .2002), whereas heavy alcohol consumption (*i.e*., ≥ 3 drink/day) is associated with an increase in risk of 40 to 50 % (Pelucchi *et al.,*2011; Seitz *et al*.,2012). In addition, high frequency of alcohol consumption is associated with increased breast cancer mortality (Allemani *et al.,* 2011).

Diets rich in fruits and vegetables are also implicated in breast cancer risk reduction. Meta-analysis showed that high intake of fruits, and fruits and vegetables combined can be associated with reduction in risk of breast cancer. Dietary fiber intake was also inversely associated with breast cancer risk (Aune *et al.,* 2012). Physical activity of vigorous intensity is associated with a greater reduction in breast cancer risk than low intensity activity and a recent study reported a more pronounced risk reduction for vigorous (26%) than moderate (22%) physical activity (Friedenreich and Cust, 2008).

Classification of breast cancer

Classification of breast cancer is often based on its receptor status, histological structure and stage. However recent advances in microarray –based gene expression profiling led to identification of molecular subtypes which can help for further individualization of breast cancer (Eroles *et al.,* 2012).

Histologically breast cancer can be divided into non-invasive (in situ) and invasive carcinomas, depending on whether or not tumour cells penetrate through the basement membrane. Non-invasive carcinomas are further divided into lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS) (Lakhani *et al.,* 2012). LCIS is considered more as a risk factor of breast cancer rather than a breast cancer precursor lesion. Invasive breast cancer can be classified into six major histological subtypes; invasive ductal, invasive lobular, mucinous, tubular, medullary and papillary. Around 75% of all invasive breast carcinoma are invasive ductal carcinomas (IDCs) (Lakhani *et al.,* 2012; Malhotra *et al.,* 2010).

TNM Staging of breast cancer

Correct staging of breast cancer patients have extraordinary importance and is determined by combining a few of the *most powerful prognostic factors.* The TNM classification system utilizes information on tumour size (T), metastases in regional lymph nodes (N) and distant metastases (M). Not only does it permit an accurate prognosis but in many cases therapeutic decision making is based largely on TNM classification (Edge *et al.,* 2010).Tumour size has a prognostic significance particularly for estrogen receptor (ER)$^-$ positive and node-negative patients. Tumour size and axillary lymph node status are in a close relationship, since it is known that larger primary tumours are more likely to develop regional lymph node metastases (Fitzgibbons *et al.,* 2001). The presence of distant metastases classifies breast cancer to stage IV and is virtually always a predictor of an incurable disease, where treatments given are mostly palliative (Nicolini *et al.,* 2006).

Prognostic factors of breast cancer

Positive steroid receptor (ER+ and PR+) status indicates a higher probability that the patient would benefit from the use of hormonal adjuvant therapy via estrogen agonists (tamoxifen) or aromatase inhibitors and also associated with low mortality and recurrences (Schiff *et al.,* 2010). The encoding gene for Human epidermal growth factor receptor 2 (HER-2) is considered to be a proto-oncogene and is found to be amplified in approximately 15% of all breast cancers. HER-2 encoding gene amplification leads to HER-2 over-expression in cancer cells which in turn results poor overall survival, higher recurrence risk and weaker response to hormonal therapy (Chang and Hilsenbeck, 2010). However, early stage as well as metastatic HER-2-positive breast cancers

are treated with a specific humanized monoclonal antibody to HER-2 (trastuzumab) which significantly reduces the risk of recurrence and mortality in these patients (Payne *et al.,* 2008; Ebstein *et al.,* 2010). Ki-67 immunohistochemistry assessed for Tumour proliferation and Proliferation has prognostic significance and is often use when evaluating the need for adjuvant therapy (Stuart-Harris *et al.,* 2008).

Treatment

Treatment of stage I–III breast cancer is based on optimal surgical removal of the primary tumor and possible metastatic lymph nodes, followed by postoperative radiation therapy and adjuvant therapies based on the risk of relapse and tumor features .The indication for adjuvant therapy is at least a 10% estimated relapse risk over 10 years of follow-up. Tamoxifen and aromatase inhibitors can be used as adjuvant therapy for hormone receptor-positive tumours and trastuzumab can be used for HER-2-positive tumours. Chemotherapy is most usually based on combinations of docetaxel, cyclophosphamide, epirubicin and 5-fluorouracil (Finnish breast cancer group, 2013).

Free radicals and cancer

Free radicals are molecules with high instability and reactivity due to the presence of an odd number of electrons in the outermost orbit of their atoms. Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are key players in the initiation and progression of tumor cells and enhance their metastatic potential, they are now considered as hallmark of cancer. These species lead to genomic damage and genetic instability, and they participate as intermediaries in mitogenic and survival signals *via* growth factor receptors and adhesion molecules, promoting cell mobility, inducing inflammation/repair and angiogenesis in the tumor microenvironment (Ríos-Arrabal *et al*., 2013).

Reactive oxygen species may produce breaks and considerable damage in the DNA molecule, producing mutations and eventually cancer (Coussens and Werb, 2002; Klaunig *et al.*, 2010). The amino acids that form proteins may also undergo alterations that modify their molecular structure, hindering their biological action. In the case of enzymes, oxidative damage may hinder their catalytic action.

Polysaccharides, which play a part in epithelium protection and/or lubrication roles, may also be affected, thereby reducing defenses and favoring inflammations (Zaremba and Olinski, 2010). Lipids, especially those containing polyunsaturated fatty acids, are especially prone to non-controlled oxidation induced by free radicals. They produce major damage in cell membranes, where these fatty acids have an essential function (Klaunig *et al*., 2010; Pande *et al*., 2011; Fuchs-Tarlovsky, 2013; Pervin *et al.,* 2013).

Recent research results have suggested that defective ROS signalling and oxidative stress could have significant roles in the carcinogenesis of breast and ovarian cancer as well as in the pathogenesis of endometriosis and polycystic ovary syndrome (PCOS) (Karihtala and Puistola, 2011, González, 2012, Worley *et al.,* 2013). In this study the serum markers of oxidative stress including lipid peroxidation product (MDA level), protein carbonyl level, uric acid level, Albumin level, reduced glutathione level, total antioxidant capacity among various clinical stages of breast cancer patients attending Tikur Anbesa specialized Teaching Hospital were investigated and compared with control groups.

1.2 Statement of the problem

Oxidative stress is characterized by increased free radical generation and/or decreased antioxidant levels in the target cells and tissues leading to damage of important biomolecules and cells. Proteins, lipids and DNA are significant targets for oxidative attack. Oxidative damage and modification of these molecules can increase the risk of mutagenesis. It has also been suggested to play an important role in breast carcinogenesis (Vera-Ramirez *et al*., 2011). Breast cancer cells are subjected to a high level of oxidative stress, both intracellular and extracellular. It has been reported that breast cancer patients suffer increased DNA damage, either independently or because breast cancer patients have impaired DNA repair mechanisms, particularly those specialized in the removal of oxidative damage and increased serum concentrations of carbonyls (Reuter *et al*., 2010).

Antioxidants are responsible for the catabolism of ROS in our cells and their adequate function is highly important for the maintenance of a strict cellular redox balance which is required to prevent the unwanted effects of ROS. However, several studies showed that endogenous antioxidant mechanisms are reduced in breast cancer patients. Enhanced ROS production and/or disturbed antioxidant function can lead to a state where ROS escape the control of antioxidants and are free to cause damaging reactions with cellular macromolecules such as proteins, lipids and DNA (Valko *et al.,* 2006).

To ensure survival, breast cancer cells must acquire special adaptive mechanisms that counteract the toxic effects of free radicals exposure. These mechanisms may involve the activation of redox-sensitive transcription factors and increased expression of antioxidant enzymes as well as anti-apoptotic proteins. Recent data revealed that different breast cancer cell types show different intracellular antioxidant capacities that may determine their ability to resist radiotherapy and chemotherapy (Vera-Ramirez *et al*., 2011).

1.3 Literatures Review

1.3.1 Reactive oxygen species (ROS) production

Reactive oxygen species are a group of highly reactive oxygen metabolites that are constantly produced in our cells to meet the needs of several crucial physiological processes such as cellular signalling, immune responses, hypoxia adaptation, and aging and wound healing (Brieger *et al.,* 2012). ROS are formed in step-by-step reactions that begin from reduction of an oxygen molecule (O_2) to a superoxide anion ($O_2^{\cdot -}$). ROS include the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$), ozone (O_3), hypohalous acids and organic peroxides (Nathan and Ding, 2010).

Many researches proved that ROS are required and generated in mammalian cells as signalling molecules in several physiological processes such as gene transcription, apoptosis and the immune response (Nathan and Cunningham-Bussel, 2013). Most ROS are generated in cells by the mitochondrial respiratory chain (Poyton, 2009).

During endogenous metabolic reactions, cells produce ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and organic peroxides as normal products of the biological reduction of molecular oxygen (Reuter *et al.,* 2010). Mitochondrial oxidative phosphorylation is one of among those several most essential and endogenous sources of ROS (Murphy, 2009). Peroxisomes constantly produce H_2O_2 in diverse redox reactions where they oxidize various metabolites such as fatty acids, purines, amino acids and also polyamines with the help of several oxidative enzymes and donate electrons directly to O_2, reducing it to H_2O_2 (Bonekamp *et al.,* 2009). The endoplasmic reticulum is a notable source of ROS via several contributors such as the flavoenzyme Ero1, diamine oxidase, cytochrome P_{450} and B_5 enzymes. Protein oxidation during protein folding in the endoplasmic reticulum is considered to produce ROS as a byproduct (Malhotra and Kaufman, 2007). Extracellular spaces and cytosol also contain ROS-producing enzymes such as xanthine oxidase and lipoxygenase, respectively (Brown and Borutaite, 2012).

Most of the endogenous sources of ROS produce either superoxide anion or hydrogen peroxide, from which other ROS are mainly derived through specific reactions with other molecules. In addition, the majority of $O_2^{\cdot-}$ is dismutated to H_2O_2 by superoxide dismutase (SOD) near the production site, since only a small proportion of extremely reactive $O_2^{\cdot-}$ is able to cross membranes, whereas more stable H_2O_2 is free to do so (Brown and Borutaite ,2012). If $O_2^{\cdot-}$ and H_2O_2 are not scavenged properly, they can form extremely reactive ·OH through the Haber–Weiss reaction, which is catalyzed by free transition metal ions such as iron, copper, chromium and cobalt (Valko *et al.,* 2006). The Haber–Weiss reaction consists of two consecutive reactions (Kehrer, 2000). In the first step, $O_2^{\cdot-}$ reduces ferric ion (Fe^{3+}) to more reactive ferrous ion (Fe^{2+}):

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2 \quad \text{..... (Equation 1)}$$

In the second reaction, called the *Fenton reaction*, Fe^{2+} reduces H_2O_2:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad \text{..... (Equation 2)}$$

The net Haber–Weiss reaction is:

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\cdot}OH \quad \text{..... (Equation 3)}$$

There also several exogenous sources contribute to the total amount of ROS to which our cells are exposed. Ionizing radiation can cause covalent bonds of intracellular H_2O to split, forming hydrogen radicals (·H) and ·OH (Kohen and Nyska, 2002).

Ultraviolet radiation also has the ability to produce ROS (Birben *et al.,* 2012). Various xenobiotics such as drugs, air pollutants and chemicals are known to increase our exposure to ROS either by directly containing them or by producing them as metabolic by-products. Food also contains significant amounts of oxidized molecules (Kohen and Nyska, 2002).

1.3.2 Catabolism of ROS (Role of Antioxidants)

Human body harbor and utilize several enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT), thioredoxin (Trx), peroxiredoxin (Prx), glutathione peroxidase (GPx) and glutathione transferase (GST). Non-enzymatic antioxidants include low-molecular-weight compounds such as vitamins C and E, glutathione (GSH), carotenoids and flavonoids which are responsible for transforming free reactive radicals into molecules that are less damaging (Birben *et al.,* 2012).

SOD is the only antioxidant capable of catalyzing the dismutation of $O_2^{\cdot-}$ to H_2O_2: The activity of SOD is based on continuous oxidation and reduction of its active site transition metal ion. Human cells have three isoforms of SOD ((SOD1 (CuZn-SOD), SOD2 (MnSOD) and SOD3 extracellular SOD (ECSOD)) which differ from each other mostly by active site metal, subcellular location and reaction rate constants (Kohen and Nyska, 2002; Valko *et al.,* 2006).

Catalase is one of the most important antioxidant enzymes scavenging H_2O_2. It is formed from four identical subunits that each contains a haem group at the active site (Birben *et al.* 2012). Catalytic breakdown of H_2O_2 is a two-step reaction where H_2O_2 first oxidizes the haem iron of catalase to form compound I, which is then used to oxidize another molecule of H_2O_2 (Kohen and Nyska, 2002). The net reaction is:

$$2\ H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(Equation 4)}$$

Glutathione peroxidase (GPx) are also an antioxidant enzyme group capable of catalyzing the reduction of H_2O_2 and other hydroperoxides. It has been suggested that the antioxidant function of GPx is crucial only under severe oxidative stress, since knock-out mice develop normally without GPx but are killed by severe acute oxidative stress (Flohé and Maiorino, 2013).

Peroxiredoxins, enzyme family of human peroxiredoxins consists of six (I–VI) distinct enzyme isoforms which all are capable of reducing peroxides such as H_2O_2 and alkyl hydroperoxides (Karihtala and Soini 2007). Prxs are comprehensively spread throughout the subcellular space, which makes them important detoxifiers of peroxides (Wood *et al.,* 2003).

Glutathione is also an important antioxidant compound responsible for maintaining intracellular redox homeostasis. This redox balance is altered under hypoxia conditions, as in the case of tumors, with the production of ROS and NO•. Glutathione exists in reduced (GSH) and oxidized (glutathione disulphide, GSSG) states (Ríos-Arrabal *et al.,* 2013). In healthy cells and tissues, over 90% of total glutathione is in reduced form GSH and less than 10% in disulphide form GSSG (Griffith, 1999).

Glutathione in its reduced state sequestrates ROS, which is transformed and recycled by the action of the glutathione-reductase enzyme (GRd). GSH is an essential cofactor for antioxidant cells known as GSH peroxidases, including GPx, which are used to detoxify peroxides, including the H_2O_2 generated in cell membranes that react with GSH (Ríos-Arrabal *et al.,* 2013).

Vitamin E (α-tocopherol) is considered to be the principal lipid soluble chain breaking antioxidant. Peroxyl radicals are scavenged by α-tocopherol in biological systems. Phenols, such as α-tocopherol, typically trap lipid peroxyl radicals during lipid peroxidation (Burton, 1983). Uric acid is the final enzymatic product in the degradation of purine nucleosides and free bases and is considered an important antioxidant contributing to the total antioxidant capacity. It can scavenge peroxyl, hydroxyl and superoxide radicals and inhibit oxidative DNA, protein, and lipids damage (Stinefelt *et al*., 2005).

1.3.3 Physiological importance of ROS

ROS participate in the regulation of several essential physiological processes, mainly through cellular signal transduction. Regarding cellular signalling, H_2O_2 is the most important member among ROS, since it fulfils best the properties of a second messenger (Forman *et al.,* 2010).

***Regulation of transcription factors**:* ROS participate in gene expression by modulating the activity of specific transcription factors and therefore regulate processes such as cell proliferation (MAPK, PI3K, PTEN, and P_{53}), apoptosis (ASK1, P53), metabolism (Shc), antioxidant function (Nrf2, Keap1, and Ref-1), the inflammatory response (NF-κP), DNA repair (ATM, Ref-1) and iron homeostasis (IRP) (Ray *et al.,* 2012). Many of these transcription factor systems are considered to have a crucial role in carcinogenesis, since cellular redox status is usually significantly altered in tumour tissue, leading to abnormal expression of redox-regulated genes and potentially offering tumour survival and growth advantage and also protection against anti-cancer treatments (Karihtala and Puistola, 2011).

***Adaptation to hypoxia**:* In hypoxic conditions, the activity of prolyl hydroxylase is known to decrease, allowing hypoxia inducible factor-1 α (HIF-1α) to stabilize and HIF-1 heterodimers to form. H_2O_2 is thought to inhibit prolyl hydroxylase by reacting with its active site non-haem iron (Murphy, 2009). Mitochondrial ROS production increases in hypoxic conditions, leading to inhibition of prolyl hydroxylase and activation of HIF-1. It would also seem that chronic hypoxia and active HIF-1 are likely to dampen mitochondrial ROS production by way of various feedback mechanisms to avoid ROS overproduction-induced damage and cell death (Sena and Chandel, 2012). Enhanced HIF-1 expression is associated with several malignancies including breast and ovarian cancer (Karihtala and Puistola, 2011).

Aging: ROS have also been suggested to have a critical role in the aging process. ROS generation and oxidative damage is increased with age and that many age-dependent diseases such as Alzheimer's, Parkinson's and carcinomas are associated with ROS derived damage (Hekimi *et al.,* 2011). In addition, mitochondrial ROS production is also known to increase with aging and to correlate negatively with the length of life (Hekimi *et al.,* 2011).

Immune function: Human immune function and response is highly dependent on ROS. Macrophages and neutrophils use NADPH oxidase to generate large amounts of ROS in the form of an "oxidative burst" that is used to eliminate microbes (Nordberg and Arnér, 2001). In addition to direct pathogen elimination, ROS also participate in the activation and function of T-lymphocytes and inflammasome, in the regulation of Toll-like and RIG-I-like receptor pathways

and in the promotion of NF-κP signalling (Brieger *et al.,* 2012). ROS are responsible for such a large amount of normal immune functions, persistent oxidative stress can cause chronic inflammation, which is known to promote most carcinogenic stages (Reuter *et al.*, 2010).

1.3.4 Deleterious potential of ROS

Excess reactive oxygen species production or insufficient antioxidant expression leads to the formation of more reactive compounds, such as •OH and $ONOO^-$ which cause damage to DNA, lipids and proteins with their non-specific reactions (Valko *et al.,* 2006). Due to their high reactivity and short half-life species such as •OH and $ONOO^-$ inflict damage in the immediate proximity of their production site. Especially •OH is able to react and oxidize any component of the DNA molecule including purines, pyrimidines and deoxyriboses (Valko *et al.,* 2004). These reactions lead to the formation of oxidized DNA adducts which are known as "footprints" of ROS-mediated DNA damage enable for detection of the activity of these extremely reactive radicals (Valko *et al.,* 2004). The most studied oxidized DNA adduct, the nucleoside 8-hydroxy-2'-deoxyguanosine (8-OHdG), is formed when •OH oxidizes the C_8 carbonyl group of guanine (Klaunig and Kamendulis, 2004). Generally, oxidative DNA damage can result in a wide variety of harmful modifications including single- and double-strand DNA breaks, DNA cross-links, deletions, frame shifts, base-free sites and chromosomal rearrangement (Valko *et al.,* 2004). To prevent potential mutagenesis, cytostasis and cytotoxicity caused by oxidative DNA damage, the removal and repair of damaged DNA lesions is extremely important for the integrity of DNA (Evans *et al.,* 2004).

Lipids, especially those containing polyunsaturated fatty acids in cell membrane phospholipids, are especially prone to non-controlled oxidation induced by free radicals. They produce major damage in cell membranes, where these fatty acids have an essential function (Cuzzocrea *et al.,* 2001). By attacking the methyl groups of polyunsaturated fatty acids, ROS such as •OH are able to initiate a chain reaction called lipid peroxidation in cell membranes (Valko *et al.,* 2004).The initial reaction is followed by the formation of lipoperoxyl radicals which can further oxidize other unsaturated fatty acid methyl groups, while reducing themselves to lipid hydroperoxides in the process (Kohen and Nyska, 2002).

Hydroperoxides form alkoxy and peroxyl radicals which finally decompose to end products of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxy-2-noneal (HNE) (Barrera, 2012). MDA and HNE are moderately reactive compounds known to have both carcinogenic and physiological effects in a concentration-dependent manner similar to ROS (Karihtala and Soini, 2007; Barrera, 2012).

Lipid peroxidation is a significant form of ROS-mediated damage because of the abundance of lipid membranes in cells and because basically one $^{\bullet}OH$ is sufficiently potent to cause the peroxidation of all polyunsaturated fatty acids in a single lipid membrane (Kohen and Nyska, 2002). Uncontrolled lipid peroxidation will eventually disturb the normal function and structure of cell membranes and increase the generation of peroxidation end products to injurious levels (Karihtala and Soini, 2007). Peroxidation of plasma membranes, which contain a high concentration of polyunsaturated fatty acids, is a mechanism leading to growth inhibition and cell death (Cerutti *et al*., 1994). Death can occur by necrosis, but lipid peroxidation can trigger the process of apoptosis, activating the intrinsic suicide pathway present within all the cells (Cerutti *et al*., 1994; Södergren, 2000).

Proteins are major targets for reactive oxidants in the cells. Protein carbonyl content is the most commonly used marker of protein oxidation (Dalle-Donne *et al*., 2003). Oxidative damage to proteins brings to the formation of protein–protein cross-linked derivatives or oxidation of amino acid side-chains, through structural changes of protein backbone and/or peptide bond cleavage, finally resulting in loss of physiological functions (Shacter, 2000).Oxidation of a protein backbone leads first to the formation of peroxyl radicals and finally to the fragmentation of backbone either *via* imine or alkoxyl radical formation (as reviewed by Davies 2005). Oxidation of side chains produces a wide variety of distinct compounds and can lead to dimerization and aggregation. These ROS-mediated modifications to protein backbones and/or side-chains eventually lead to functional and structural abnormalities (Davies, 2005). Majority of other forms of oxidative damage and modification to proteins seem to be irreversible, while the oxidation of thiol groups of cys residues in ROS-mediated signalling is easily reducible by thiol-based antioxidants (Davies, 2005).

Protein carbonyls are formed because of the oxidative modifications of proteins. Carbonyl groups are introduced into proteins by two distinct mechanisms: oxidative (direct) and non-oxidative (indirect). Oxidative mechanisms, which are metal catalyzed, involve the direct reaction of certain reactive oxygen species (e.g., hydrogen peroxide and lipid hydroperoxides) with protein side chains (Dkhar and Sharma, 2011). Damage of proteins by oxidative stress could be involved in inflammation-related carcinogenesis (Beal, 2000; Ellis, 2007). Carbonyl groups are formed during the oxidation of protein side chains, resulting in chemically stable products which serve as useful markers for assessing oxidative stress in vivo (Shacter,2000); their accumulation in biological fluids has been observed in several pathological processes (Beal,2000; Ellis.,2007). Detection of elevated levels of protein carbonyls in blood or tissues indicates generally a disease associated dysfunction and defective immunological responses and macrophages functions (Thanan *et al.,* 2012). In particular, plasma/serum protein carbonyl levels were significantly higher in breast cancer patients than in healthy women (Rossner *et al.,* 2007; Tesarova *et al*., 2007) suggesting that enhanced protein carbonyl concentrations are significantly associated with increased breast cancer risk (Rossner *et al.,* 2007).

1.3.5 Reactive Oxygen Species in Carcinogenesis

It is well documented that high concentrations of ROS act in multiple signaling cascades related to various behaviors in cancer cell, such as survival, proliferation, angiogenesis and metastasis. ROS are thus considered responsible for initiation, development, progression, invasion and metastasis of cancers. Clinical evidence has implicated the fundamental role of ROS in invasion and migration (Echiburú-chau *et al.,* 2011).

ROS contribute in different ways to carcinogenesis and the malignant progression of tumor cells, enhancing their metastatic potential. And they also participate as intermediaries in mitogenic and survival signals via growth factor receptors and adhesion molecules, promoting cell mobility, inducing inflammation/repair and angiogenesis in the tumor microenvironment (Ríos-Arrabal *et al*., 2013). Carcinogenesis is a three-stage process which is firstly initiated by a non-lethal DNA mutation in a single cell, promoted by proliferative and anti-apoptotic stimuli, finally progressing to a neoplastic tumour with uncontrolled growth and invasion potency (Valko *et al.,* 2006).

Mammalian cells suffering from oxidative stress have either excessive amounts of ROS or diminished antioxidant activity that leads to the formation of more damaging ROS such as $^{\bullet}OH$ (Valko *et al.,* 2006) . DNA reacting with $^{\bullet}OH$ can cause point mutations that activate oncogenes such as *c-fos*, *c-jun*, *c-myc*, *c-raf1* and *K-Ras* or inactivate tumour suppressor genes such as *p53* (Karihtala and Puistola, 2011). In addition, $^{\bullet}OH$ can further prevent the proper repair of damaged DNA by reacting with DNA repair and synthesizing enzymes (Liou and Storz, 2010). ROS-derived mutation potential of oxidative stress is likely to prevail through the different stages of carcinogenesis depending on the redox state of tumour cells, but it is likely that these mutations play their most significant role in the initiation stage (Halliwell, 2007).

ROS are considered to have important roles as signalling molecules, promoting proliferative and anti-apoptotic pathways. Human cancer cells are known to have amplified ROS production due to the several factors such as accelerated metabolism, increased activity of peroxisomes and phagocytes (oxidative bursts and cytokine secretion), constant change between reperfusion and hypoxia, mitochondrial dysfunction and deviant growth factor (PDGF, EGF, insulin), cytokine (IFNγ, TNFα, IL-1, TGFβ) and enzyme (NOX, COX, LOX, K-Ras, rac-1) activity (Liou and Storz, 2010, Karihtala and Soini ,2007, Fiaschi and Chiarugi ,2012).

In addition to this, amplified ROS production, studies also revealed that ROS-mediated transcription factors such as NF-κB, nrf-2, HIF, Erk1/2, AP-1 and Akt to be up-regulated in malignant tumours, promoting cell growth, cell survival, inflammation and metabolism (Liou and Storz, 2010). ROS are also closely related to angiogenesis and metastasis by way of increasing the release of VEGF and angiopoietin, promoting anchorage-independent growth, inhibiting anoikis and regulating matrix metalloproteinases, Snail, src and TGF-β to induce epithelial-mesenchymal transition (Cui, 2012; Fiaschi and Chiarugi, 2012). Tumour supportive effects of reactive oxygen species are most likely to be achieved with low to moderate cellular levels of ROS. Overly high levels of ROS are known to promote cell cycle arrest and apoptosis through the activity of various proteins such as Shc, FOXO, p53 and Ask-1 (Liou and Storz, 2010). An extremely high level of oxidative stress can lead to necrosis (Valko *et al*., 2006, Liou and Storz, 2010).

Augmented antioxidant activity is known to prevent ROS-promoted cell death (Valko *et al.*, 2006). Increased antioxidant levels have been observed in several malignancies including breast cancer and ovarian cancer and are often related to the activation of some ROS signalling pathways such as those involving NF-κB, Nrf-2 and HIF (Liou and Storz ,2010 ; Karihtala and Puistola, 2011).

Reactive oxygen species are also partly responsible for the shift towards glycolytic metabolism (Warburg effect) in tumor tissues via HIF, ras, myc and p53, which is also known to promote antioxidant production (Fiaschi and Chiarugi, 2012). Amplified antioxidant function, as well as the activation of these signalling pathways in tumor tissues, have also been linked to the development of resistance against radiotherapy and chemotherapy (Reuter *et al*., 2010). Therapies are largely based on ROS-mediated cell death (Karihtala and Puistola, 2011). Recent studies indicated that high survivability of cancer stem cells, a distinct cancer cell population that has also been isolated from breast and ovarian carcinomas and suggested to have roles in cancer relapse and metastasis could be based on the up-regulation of antioxidants (Reuter *et al*., 2010).

1.3.6 Roles of oxidative stress in breast cancer

1.3.6.1 Oxidative Stress in breast cancer microenvironment

Breast cancer microenvironment system comprises of breast cancer cells, fibroblasts, adipocytes, immune, and endothelial cells and interactions between cancer cells and stroma regulate breast cancer pathways (Drutel *et al.,* 2013). Among the many factors influencing development, progression, and metastasis, oxidative stress has an important role in the initiation and preservation of breast cancer progression. One way in which cellular ROS impact cell signaling is through their localized accumulation. ROS byproducts of the electron transport chain in mitochondria or activation of the NADPH oxidases (NOX) function in all cell types, including both breast carcinoma and cancer stroma. Stromal-derived NOX4 - ROS are able to stimulate migration of MCF-7 cells in a paracrine manner (Hecker *et al*., 2009; Sampson *et al*., 2011; Tobar *et al*., 2010).

Overexpression of NOX4 in normal breast epithelial cells results in cellular senescence, resistance to apoptosis, and tumorigenic transformation, as well as increased aggressiveness of breast cancer cells (Graham *et al*., 2010).

Several recent studies have confirmed that ROS, including hydrogen peroxide, alone are able to drive the differentiation of normal fibroblasts into myofibroblasts, which are able to generate high amounts of hydrogen peroxide themselves, increasing oxidative stress in the microenvironment (Comito *et al.,* 2012;Toullec *et al*., 2010). Almost 80% of fibroblasts acquire an activated phenotype in breast cancer (Kalluri and Zeisberg, 2006). In addition, type I collagen secreted by myofibroblasts causes higher breast density, which contributes to mammary tumor formation and metastasis (Provenzano *et al*., 2008). Furthermore, type I collagen contributes to decreased chemotherapeutic agent uptake and altered tumor cell sensitivity to a variety of chemotherapies.

Secreted metalloproteinases (MMP) such as MMP-2, MMP-3, and MMP-9 increase extracellular matrix turnover and are themselves activated by oxidative stress (Fu *et al*., 2001; Koch *et al*., 2009; Provenzano *et al*., 2008; Radisky *et al*., 2005). Additionally growth factors such as transforming growth factor (TGFβ), insulin-like growth factor (IGF), tumor necrosis factor-α (TNFα), or platelet-derived growth factor (PDGF), stimulate ROS production through NOX. Once activated, the tumor microenvironment network produces large amounts of ROS, initiating tumor growth. It would be clinically advantageous to detect this large ROS activity early enough to prevent further cancer progression (Drutel *et al.,* 2013).

Recent studies show that in the breast cancer microenvironment, oxidative stress causes mitochondrial dysfunction, which manifests itself by the upregulation of numerous factors, such as the nuclear respiratory factor 1 (NRF1), which was shown to be upregulate in cancer stroma due to oxidative stress (Witkiewicz *et al*., 2011). The mtDNA is extremely sensitive to oxidative damage due to the lack of protective histones and efficient repair mechanisms located in the nucleus. This is the mechanism by which excess mitochondrial ROS promotes malignant cell transformation (Witkiewicz *et al*., 2011).

Reactive oxygen species can damage DNA and the division of cells with unpaired or misrepaired damage leads to mutations. The majority of mutations induced by ROS appear to involve modification of guanine, causing G→T transversions (Lunec *et al*., 2002; Waris and Ahsan, 2006). If it relates to critical genes such as oncogenes or tumor suppressor genes, initiation/progression can result (Ames et al., 1993).

Mutations caused by oxidative DNA damage include a range of specifically oxidized purines and pyrimidines, alkali labile sites, single strand breaks and instability formed directly or by repair processes (Dizdaroglu *et al*., 2002; Cooke *et al*., 2003; Waris and Ahsan, 2006). Because of the multiplicity of DNA modifications produced by ROS, it has been difficult to establish the frequency and specificity of mutations by individual oxygen radical induced lesions. Some of these modified bases have been found to possess mutagenic properties. Therefore, if not repaired they can lead to carcinogenesis. Studies show that although all the four bases are modified by ROS, mutations are usually related to modification of GC base pairs, while that of AT base pair rarely leads to mutations (Waris and Ahsan, 2006).

Oxidative DNA damage may be involved in the development of breast cancer. Increased steady-state levels of DNA base damage with a pattern characteristic of .OH attack have been reported in inflammatory breast disease where malignant progression can occur. It is reported that elevated levels of 8-oxo-dG adducts in DNA play a fundamental role in breast cancer. Evidence also exists for the progression of breast tumor to the metastatic state and is an important etiologic factor (Malins *et al*., 1996; Waris and Ahsan, 2006).

In breast cancer, estrogen exposure and its hydroxylated metabolites seem to increase reactive oxygen species (ROS) production by regulating mitochondrial function and bringing about lipid peroxidation (Vera-Ramirez *et al.,* 2011). It indicated that the carcinogenic effects of estrogen exposure may be explained by ROS-derived genomic instability and activation of tumourigenic signalling pathways (involving NF-κB, nrf-2, AP-1) (Okoh *et al.,* 2011). Lipid peroxidation markers levels of MDA and 4-HNE, are elevated in breast cancer tissue and in the serum and urine of breast cancer patients when compared with healthy controls (Karihtala *et al.,* 2011, Vera-Ramirez *et al.,* 2011; Pande *et al.,* 2012).

1.3.6.2 Reactive Oxygen Species as signaling intermediary in breast cancer

Oxidative stress increase mutation rate and accelerate tumor progression

Reactive oxygen species are one of the most powerful DNA damaging agents. ROS cause strand breaks, alterations in guanine and thymine bases, and sister chromatid exchanges this in turn inactivate tumour suppressor genes within tumour cells, or further increase expression of proto-oncogenes. Genetic instability due to persistent oxidative stress will then increase the malignant potential of the tumor (Brown and Bicknell, 2001).The Janus kinase (JNK) pathway is also involved in responses related to oxidative stress, hence controlling oncogenic expression, and it also mediates in cell death by p53-induced apoptosis (Schramek *et al*., 2011; Raj *et al*., 2012). JNK inhibition produces changes in senescence and causes a rapid increase in ROS production in the mitochondria and in the response to DNA damage in breast carcinoma cells (MCF-7). This ROS production is attributed to the suppression of Bcl-2 (B cells of lymphoma 2) phosphorylation, causing DNA damage and stimulating the activation of p-53 (Lee *et al*., 2009).

Oxidative stress activates growth-promoting signalling pathways

Sub lethal oxidative stress promotes cell proliferation *in vitro*, with both superoxide and hydrogen peroxide stimulating growth. Proliferation in response to H_2O_2 may be due to the activation of mitogen-activated protein kinases (MAPKs) (Burdon, 1995). HeLa cells treated with H_2O_2 undergo a sustained activation of all three MAPK pathways (Xiantao *et al*., 1998): extracellular signal related protein kinase; c-Jun amino-terminal kinase/stress-activated protein kinase; and p38. Hyperphosphorylation of c-Jun by oxidative stress activates activator protein-1 in MCF-7 breast carcinoma cells, a response that stimulates proliferation (Schiff *et al*., 2000), and multidrug-resistant human breast carcinoma cells rapidly activate extracellular signal related protein kinase-2 when stressed by glucose deprivation (Lee *et al*., 1998). Furthermore, ROS may trigger mitosis via MAPK independent mechanisms. Oncogenic *Ras* causes ROS production by activating Rac1 and the NADPH-oxidase. In *Ras*-transformed human fibroblasts, ROS drive cell cycle progression without the activation of MAPK pathways (as reviewed by Brown and Bicknell, 2001).

Reactive oxygen species acts on the growth factor stimulus after receptor tyrosine kinases (RTKs) and small GTP proteins; such as Ras and Rac-, and before MAPK members. Ras and Rac proteins appear to be directly related to the production of superoxide anions and therefore to cell transformation ((Ríos-Arrabal *et al.,* 2013). The Janus kinase (JNK) pathway is involved in responses related to oxidative stress, hence controlling oncogenic expression, and it also mediates in cell death by p53-induced apoptosis (Schramek *et al*., 2011; Raj *et al*., 2012). JNK inhibition produces changes in senescence and causes a rapid increase in ROS production in the mitochondria and in the response to DNA damage in breast carcinoma cells (MCF-7). This ROS production is attributed to the suppression of Bcl-2 (B cells of lymphoma 2) phosphorylation, causing DNA damage and stimulating the activation of p53 (Lee *et al*., 2009). Activation of the JNK signaling pathway involves an anti-tumorigenic response, controlling the oncogene expression. This response is related to the activation of oncogenes that depend on oxidative stress and are controlled by p53 (Schramek *et al*., 2011).

ROS also intervene in the regulation of the PI3K/AKT (phosphatidyl inositol 3-kinase/protein kinase) pathway, which is related to cell growth, survival, and the activation of transcription factors, e.g., hypoxia inducible factors (HIFs) (Weinberg and Chandel, 2009).Nutritional deficit leads to an ROS increase that eventually affects mTOR via the PI3K/ AKT pathway, thereby activating autophagy. PI3K is altered in different tumor types, with consequent effects on the autophagic process (Shouval and Elazar, 2007; Wong *et al*., 2010).

Oxidative stress increases blood supply to tumor cells

Oxidative stress may also increase the blood supply to breast carcinoma by triggering vasodilatation. Hydrogen peroxide induces inducible nitric oxide synthase in cytokine stimulated pleural mesothelium cells, raising the possibility that oxidatively stressed breast tumour cells might show increased expression of inducible NOS (Chandel *et al*., 2000; Richard *et al*., 2000).
The nitric oxide produced would activate cGMP within nearby smooth muscle cells, leading to vasodilatation. Vasodilatation could also be triggered by carbon monoxide, because oxidative stress powerfully induces heme oxygenase-1which degrades heme to biliverdin and carbon monoxide (CO, like nitric oxide, activates cGMP) (Brown *et al*., 2000).

Oxidative stress increase risk of cancer metastasis

Blood vessel growth within the breast tumour microenvironment increases the risk of blood-borne metastasis. Angiogenesis may also promote lymphatic dissemination, a common occurrence in breast carcinoma, by elevating tumour interstitial pressure. These are not the only mechanisms by which oxidative stress can aid tumour spread, however. Oxygen radicals may also increase tumour cell migration, increasing the risk of invasion and metastasis. Oxidative stress within breast tumours may facilitate invasion and metastasis by activating MMPs and inhibiting antiproteases. MMP-2 is a gelatinase that is believed to play a major role in breast cancer invasion and metastasis. High levels of MMP-2 correlate with poor prognosis in breast cancer patients (Duffy *et al*.,2000) and active MMP-2 is detected more frequently in malignant than in benign breast tumours. Reactive oxygen species have been shown to activate MMP-2, possibly by the reaction of oxygen radicals with thiol groups within MMP-2 (Duffy *et al*., 2000) ,Protease inhibitors, such as α1-proteinase inhibitor and plasminogen activator inhibitor, may be inactivated by oxidation of methionine residues at their active sites This facilitates the activity of various proteases, increasing invasion and the likelihood of metastasis plasminogen activator is believed to play a role in metastasis (as review by Brown and Bicknell, 2001).

Oxidative stress results in increased resistance to anti-cancer therapy

Severe oxidative stress leads to apoptosis. On the other hand, persistent oxidative stress at sub lethal levels may cause resistance to apoptosis. The induction of programmed cell death by ROS is dependent on p53 in human cell lines (Lazo *et al*., 1998). Constitutive oxidative stress within breast carcinoma cells may accelerate the selection of p53 knockout tumour cell clones, which have an apoptosis resistant phenotype. The antioxidant thiols thioredoxin and metallothionein in are rapidly upregulate in response to oxidative stress (Brown and Bicknell, 2001), and the antioxidants, SOD, glutathione peroxidase and catalase show increased expression or activity in breast tumour tissue as compared with normal controls (Portakal *et al*., 2000). An upregulation of anti-ROS defences in cancer cells may explain why tumour cell lines *in vitro* are extremely resistant to cytolysis by hydrogen peroxide. Moreover, anti-apoptotic Akt (protein kinase B) is activated by hydrogen peroxide (Brown and Bicknell, 2001).

An anti-apoptotic response to chronic oxidative stress may have severe implications for anticancer therapy. As explained above, radiotherapy, photodynamic therapy and many chemotherapies generate oxygen radicals. Their antitumour activity is to a degree dependent on the induction of tumour cell apoptosis in response to oxidative stress and oxygen radical induced DNA damage. Persistent oxidative stress within carcinoma cells may therefore cause resistance to therapy. Oxygen radicals might also increase drug resistance by increasing carcinoma cell expression of P-glycoprotein, the multidrug-resistance efflux pump (Yokomizo *et al*., 1995; Ziemann *et al*., 1999; Brown and Bicknell, 2001).

1.4 Hypothesis

There is no difference on serum biomarkers of oxidative stress between breast cancer patients and healthy control subjects.

1.5 Significance of the study

The finding of our study could help us to show the status of serum oxidants and antioxidants markers among breast cancer patients with respect to healthy controls, as well as in relation to socio-demographic and clinical characterstics (such as clinical stage, tumor size, histologic grade, etc). This, in turn, may help to see how Ethiopian breast cancer patients are behaving in terms of oxidative stress to the disease. The outcome of the study could also be an important input to see the feasibility of testing the oxidative stress status as potential diagnostic modality so that progression of the disease may be diagnosed and followed together with other diagnostic modalities.

The outcome of the study could also help physicians to encourage assessment of those oxidative stress biomarkers before and after anti-cancer treatment which might be beneficial to improve the survival of patients with breast cancer. Furthermore, the output of this study can serve as a baseline for further studies in assessing the serum biomarkers of oxidative stress of breast cancer patients in Ethiopia.

2. Objectives of the study

2.1 General objective

- The objective of this study was to investigate the common serum markers of oxidative stress among breast cancer patients in Tikur Anbesa specialized teaching hospital, Addis Ababa, Ethiopia.

2. 2 Specific objectives

- To evaluate intensity of malondialdehyde (MDA) level among breast cancer patients.
- To evaluate the protein carbonyl level among breast cancer patients.
- To evaluate total anti-oxidant capacity (TAC) among breast cancer patients
- To evaluate reduced glutathione levels (GSH) among breast cancer patients
- To evaluate uric acid level among breast cancer patients
- To evaluate albumin level among breast cancer patients
- To correlate socio-demographic and clinical characteristics with markers of oxidative stress among breast cancer patients.

3. Participants and methods

3.1 Study area and period

The study was conducted in Tikur Anbesa specialized teaching hospital, from January 22, 2015 to April 23, 2015. Tikur Anbessa Specialized Hospital is a largest general public referral hospital in Ethiopia and sees approximately 370,000- 400,000 patients a year. The hospital opened in 1972, and is a referral hospital with 700 beds. In 1998 it was transferred to the School of Medicine by the Federal Ministry of Health (MoH), and since then it has become a University teaching hospital. The hospital is the largest teaching hospital for Addis Ababa University, School of Medicine in Ethiopia. Tikur Anbessa Specialized Hospital is now the main teaching hospital for both clinical and preclinical training of most disciplines. It is also an institution where specialized clinical services, that are not available in other public or private institutions, are rendered to the whole nation. Over 200 physicians and 627 nurses are currently working in Tikur Anbessa specialized hospital. According to the report of the Ethiopian Ministry of Health in 2010, the hospital has 700 beds with over 300,000 annual patient visits.

3.2 Study design

A prospective comparative cross sectional study design was employed involving breast cancer patients and healthy control subjects.

3.3 Population

3.3.1 Source population

Patients: All breast cancer patients who presented themselves to Tikur Anbesa specialized hospital for breast cancer diagnosis and treatment

Controls: All patients who presented themselves to "*Atena Tera Lomi Meda"* health center for medical care were considered as source population.

3.3.2 Study population

Patients: The study population was all pathologically (fine needle aspirate (FNA) OR excision biopsy) confirmed breast cancer patients fulfilling inclusion criteria, during the study period.

Controls: All clients of the *Atena Tera Lomi Meda* health center who came for family planning service, fulfilling all the following inclusion criteria during the study period.

Inclusion criteria

- ✓ Patients who are capable and willing to provide signed voluntary informed consent to participate and give blood sample for the purpose of the study.
- ✓ Women with age between 18 and 75 years.
- ✓ Women with early stage, locally advanced and metastatic (advanced) breast cancer
- ✓ Women with a Karnofsky's performance score of at least 70% (at diagnosis)

Exclusion criteria

- Pregnant
- Had previous history of cancer,
- Smoker or had previous history of smoking
- Patients or clients with other chronic conditions (DM, HTN, HIV/AIDS, rheumatoid arthritis, liver and renal impairment, etc).
- Patients who had breast surgical treatment (*i.e.*, undergone modified radical mastectomy)

3.4 Variables

Dependent variables

- Serum malondialdehyde (MDA) levels
- Serum protein carbonyl level
- Serum total antioxidant capacity
- Serum reduced glutathione (GSH) level
- Serum uric acid level
- Serum albumin level

Independent variable

- Age
- BMI
- Nutritional habit
- Alcohol use
- Physical exercise habit
- Menopausal status
- Clinical stages of breast cancer
- Tumor size

3.5 Sample size determination and sampling technique

The sample size required for the study was determined using OpenEpi software version 3.03. The usual expectations for a cross sectional study is 95% confidence (two sided confidence level) and 80% power (to detect a difference in the underlying population). The sample size required for the study was determined using the formula for single population proportion (confidence interval approach) (**Dean *et al*., 2015)**. The sample size, n, for 95% two-sided confidence interval for a proportion would be:

$$n = \frac{(Z_{1-\alpha/2})^2 pq}{d^2}$$

Where;

n = required sample size

$Z_{1-\alpha/2}$ = the $100(1-\alpha/2)^{th}$ percentile of the normal (or Gaussian) distribution. For the commonly used two-sided 95% confidence interval, $Z_{1-\alpha/2} = 1.96$,

p = Estimated proportion rate breast cancer

According to the Addis Ababa cancer registry (AACR, 2014), the proportion of breast cancer cases account for about 33%, Thus, we would use *p* to be 33% = 0.33 and thus,

q = 1 – p = 0.67

d = the margin of confidence interval = 0.05

Accordingly, $n = ((1.96)^2 \times 0.5 \times 0.5)/(0.05)^2 = \mathbf{384}$.

Considering the data from Addis Ababa cancer registry (AACR, 2014), the size of target population was N = 5701, which is less than 10,000 and hence making for finite population correction; the adjusted number of subjects, n_f, needed for the study will be calculated as:

$$n_f = \frac{n}{1 + n/N}$$

Where; n_f = the adjusted sample size

n = unadjusted sample size = 384

N = the size of target population = 988

And thus, n_f = **321**

Proportional allocation was used determine the required sample for different stages of the disease. Assuming the proportion of cases to controls to be 5:1, then 64 (20%) control participants and 257 (80%) patients with breast cancer was planned to be included. Thus, 64 patients were planned to be included in stages I, II, III and IV cases each, proportionally.

3.6 Reagents and Equipment

Chemicals and reagents

Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), Reduced glutathione (GSH), 2,2 -azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid),sodium acetate, reagent-grade glacial acetic acid, Hydrogen peroxide (H_2O_2), potassium phosphate (monobasic and dibasic), all from Sigma-Aldrich Co (St. Louis, MO, USA), were purchased from local commercial suppliers and used as received. Protein Carbonyl Hydrochloric Acid (HCl), 2,4-dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA) Solution, Guanidine Hydrochloride (GdmCl), Ethanol, Ethyl Acetate (all from Cayman chemical company (Ann Arbor, MI, USA)), were also purchased from local commercial suppliers and used as received.

Equipments

Fully automated Microplate Reader, Labtech LT-4000 (380 – 750nm, China), fully automated Mindray (BS-200E Chemistry Analyzer), Solar Spectrofluorimeter CM 2203 (220-1100 nm, Russia)**,** vortex mixer XH-R (Jiangsu, China), Eppendorf 5417R Refrigerating Microcentrifuge (Hamburg, Germany), were used.

3.7 Methods

3.7.1 Data collection

Patients or clients were requested by trained nurses to participate in the study. After informed consent is granted, data on patient and volunteer controls socio-demographic (age, weight, and nutritional, alcohol drinking, physical exercise habits) were recorded. Moreover for case groups additional clinical information (site of lesion, the histological grade, tumor size, vascular invasion, clinical stage of breast cancer were abstracted from patient medical charts or *via* interview of each participant.

The questionnaire comprised of two main characteristics: socio-demographic characteristics (including age, region, residence area, education level, marital status, height and weight) and clinico-pathological characteristics. Information was also collected on known and suspected risk factors for breast cancer and study parameters, including alcohol intake, menstrual and reproductive histories, oral contraceptive use, physical activity, prior medical history and other study variables.

During the interview, height and weight were measured using standard procedures to obtain body mass index (BMI). The participants were then classified according to categories defined by the Center of Disease Control (CDC) as "underweight" (BMI below 18.5 kg/m^2), "normal" (BMI from 18.5 kg/m^2 to 24.9 $kg/m^{2)}$ "overweight" (BMI from 25.0 to 29.9 $kg/m^{2)}$ and "obese" (BMI at or above 30.0 kg/m^2).

Nutritional habit was assessed using a food frequency question, food items classified into two groups: high fat diets (including animal fat, dairy products include butter, oils, high-fat meat) and vegetables and fruits (enriched in vitamins like vitamin D, C etc). For those food item groups the participants were asked how often they had consumed that food within a weak. Physical exercise habit was also assessed using frequency questions, participants were asked how often they perform physical exercise (including *walking, hiking, jogging, running. swimming* up to strenuous physical exercises). The questionnaire was adopted from Million Women Study Collaborative Group, 1999 with some modifications and translated to local languages.

3.7.2 Blood sampling and treatment

Five milliliter of blood samples were collected by venous arm puncture using a Vacutainer tube from both breast cancer patients and control subjects. The blood samples were centrifuged at 3000g for 10 min, and then the separated serum was stored at -80^0C until analysis.

3.7.3 Biochemical analysis of serum oxidative stress markers

3.7.3.1 Estimation of Malondialdehyde (MDA) level

***Principle*:** Lipid peroxidation was determined as described by Ohkawa *et al*., (1979). The method is based on the formation of a pink chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with Malondialdehyde (MDA) and other breakdown products of peroxidized lipid collectively called as thiobarbituric acid reactive substance (TBARS).

***Procedure*:** In a 2ml test tube 0.5 ml of serum sample was mixed with 50 µl of 2% butylated hydroxytoluene (to prevent lipid oxidation during the assay), 0.5ml of normal saline, 1ml of 20% TCA and 0.25ml of TBA reagent (200mg of TCA in 30ml distilled water and 30 ml of acetic acid) were added. The blank was prepared for each sample by substituting the TBA solution with distilled water (sample blank). The test tubes were kept for boiling at 95^0C for one hour. To each of the test tubes, 300ml of n-butanol was added and mixed well with vortex mixer.

The test tubes were centrifuged at 3000 rpm for 10 min. The separated butanol layer was collected and read in a spectrophotometer against blank at 535 nm using Solar Spectrofluorimeter CM 2203 (220-1100 nm). The TBARS concentration was calculated using the following formula and expressed in terms of nmol of MDA /ml of serum (using extinction coefficient =1.56 x 10^5). The lipid peroxidation was calculated using the formula:

nmol of MDA /ml of serum = *O. D. of sample* × *Dilution factor /E* × *volume of aliquot*

3.7.3.2 Estimation of Protein Carbonyl

Principle: Protein carbonyl was determined according to Levine *et al* (1994) method. In this method, protein carbonyls in protein samples are derivatized with DNPH. The proteins are then TCA precipitated and the free DNPH is removed by washing the protein pellet. After dissolving the protein pellet in GuHCl, the absorbance of protein-hydrozone is measured at 380 nm, and the protein carbonyl is determined.

Procedure: The 200 µl of serum sample was transferred to two 2 ml plastic tubes. One tube was the sample tube and the other was the control sample. Serum was diluted 1:40 with phosphate-buffered saline (PBS) containing 10 mM sodium phosphate, pH 7.4, and 0.14 M NaCl, and this was centrifuged twice (10 min at 11.4 000rpm in microcentrifuge) to eliminate all particulate matter that might interfere with the reaction. The diluted proteins are precipitated with cold trichloroacetic acid (TCA, 20% final concentration) and then collected by centrifugation for 10 min. A solution of 10 mM DNPH in 2 N HCl was added to the protein pellet of each sample to give a final protein concentration of 1–2 mg/ml, with 2N HCl only added to corresponding control sample. Samples were allowed to stand in the dark at room temperature for 1 h with vortexing every 15 min; they are then precipitated with 10 % TCA (final concentration) and centrifuged for 10 min. The supernatants were discarded and the protein pellets were washed once more with 10% TCA, and then washed three times with 1ml portions of ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. After the final wash, the protein pellets were resuspended in 500 µl of guanidine hydrochloride by vortexing. Both samples were then resuspended in 500 µl of 6M guanidine hydrochloride (GdmCl) for 10 min with vortex mixing.

And 220 µl of supernatant was transferred from the sample tube to two wells in duplicate of the 96-well plate. The same amount (220 µl) supernatant was also transferred from the control tube to two wells of the 96-well plate. Finally, the absorbance was measured at a wavelength between 385nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} on a plate reader. Protein carbonyl concentration was calculated using the average absorbance of each sample and control. The corrected absorbance (C_A) was obtained by subtracting the average absorbance of the controls from the average absorbance of the samples. The concentration of the carbonyls was determined by inserting the corrected absorbance into the following equation:

*Protein Carbonyl (nmol/ml) = [(CA)/ (*0.011 µM-1)] (500 µl/200 µl)*

3.7.3.3 Determination of serum reduced glutathione (GSH)

***Principle*:** Reduced glutathione (GSH) was determined by using the method of Ellman (1959). The DTNB-Thiols assay measures sulfhydryl groups with the thiol reagent 5, 5-dithiobis [2-nitrobenzoic acid] (DTNB), which forms the 5-thionitrobenzoic acid and a mixed disulfide. Under conditions of oxidative stress, free sulfhydryls decrease and disulfides increase.

Procedure: Serum reduced glutathione (GSH) levels were determined by the method of Ellman (1959). A 100µL filtered serum was diluted with 1mL of distilled water. And 500µL of the diluted serum was taken and to this 2mL of phosphate solution and 250µL of DTNB [5, 5-Dithiobis (2 nitrobenzoic acid)] reagent were added. Simultaneously, blank was maintained containing 200µL of distilled water, 300µL of precipitating solution, 2mL of phosphate solution and 250µL of DTNB. The intensity of yellow colour formed was spectrophotometrically read at 412nm against blank using *Solar Spectrofluorimeter CM 2203 (220-1100 nm, Russia)***.** The optical densities obtained were plotted against the standard graph (as indicated in annex VII). The concentration of glutathione was calculated graphically and the reduced glutathione in the serum was expressed as µM.

3.7.3.4 Estimation of Total antioxidant capacity

Principle: Serum TAC was determined by the method of Erel, O (2004).The reduced ABTS molecule is oxidized to $ABTS^+$ using hydrogen peroxide alone in 30mmol/l acetate buffer (pH 3.6). In the acetate buffer solution, the concentrate (deep green) $ABTS^+$ molecules stay more stable for a long time. While it is diluted with a more concentrated acetate buffer solution at high pH values (the acetate buffer 0.4 mol/l pH 5.8), the color is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related with the TAC of the sample. The reaction rate is calibrated with Trolox, which was used as a standard for TAC.

***Assay procedure*:** Total Antioxidant Capacity of serum was measured by adding 200 µL reagent 1 (0.4 mol/l acetate buffer solution (pH 5.8) to 5 µL serum sample and then 20 µL of Reagent 2 (the $ABTSS^+$ in acetate buffer 30 mmol/l pH 3.6) was added. Absorbance was measured using fully automated Microplate Reader, Labtech LT-4000) at wavelength of 660 nm. The first absorbance was taken before mixing of R1 and R2 (as sample blank) and the last absorbance was taken after the end of incubation period (five minutes of mixing of R1 and R2). The optical densities obtained were plotted against the standard graph (as indicated in annex VII) and results were expressed in mmol Trolox equivalent/l.

3.7.3.5 Determination of serum uric acid

Serum uric acid levels were assessed using commercial kits *Human* with the *fully automated Mindray (BS-200E Chemistry* Analyzer).

Principle and method: The current method for serum uric acid determination employs by Trinder (Barham & Trinder, 1972; Trinder, 1969). Uric acid concentration was measured by the direct enzymatic method, in which uric acid was oxidized by uricase coupled with peroxidase. Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen per-oxide formed further reacts with a 3, 5-dichloro-2-hydrobenzen sulfonic acid (DCHBS) and 4-aminoantipyrine (PAP) by the catalytic action of peroxidase to form a red colored quinine imine dye complex.

The colour intensity formed is directly proportional to the amount of uric acid present in the sample. Results are expressed in mg/dL.

$$\text{Uric acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{Allatonin} + CO_2 + H_2O_2 \quad \ldots\ldots\ldots\ldots \text{Equation 4}$$

$$2\,H_2O_2 + \text{DCHBS} + \text{PAP} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + HCl + H_2O \quad \ldots\ldots\ldots \text{Equation 5}$$

3.7.3.6 Determination of serum albumin

Serum albumin levels were assessed using commercial kits *linear* with the *fully automated Mindray (BS-200E Chemistry Analyzer).* ***Principle and methods*: -** The current method for serum albumin determination employs by (Doumas *et al*., 1971; Tietz., 1978) .The method is based on the specific binding of bromocresol green (BCG), an anionic dye, and the protein at acidic pH with the resulting shift in the absorption wavelength of the complex. The intensity of the color formed is proportional to the concentration of albumin in the sample. Results are expressed in mg/dL.

$$\text{BCG} + \text{Albumin} \xrightarrow{\text{pH 4.3}} \text{BCG - albumin complex} \quad \ldots\ldots\ldots\ldots\ldots \text{Equation 6}$$

3.8 Data quality management plans

The data collectors were trained clinical nurses and they were trained about data collection procedure and during the data collection the patient history and other information from the patients' medical record cards were cross checked. The English version of the questionnaire was translated into Amharic.

3. 9 Ethical consideration

The study was conducted after ethical approval is granted from the scientific and ethical committee of Biochemistry department, College of Health Sciences, Addis Ababa University after review conducted with meeting number DRERC 04/14 attended by the main researcher committee and give approval with protocol number of M.Sc. Thesis 16/14. The purpose and procedures of the study was explained to the study participants. Written informed consent was obtained from individual participants. All the information obtained was kept confidential. The right to participate or to withdraw from the study or not to participate was respected.

3.10 Statistical analysis used

The socio-demographic and clinical data collected as well as assay results were entered into SPSS version 21.0 for windows. Descriptive statistics were run to explore the socio-demographic, clinical and assay results. Continuous variables (MDA level, protein carbonyl level reduced glutathione levels, total anti-oxidant capacity albumin level uric acid level, were expressed as mean (with standard deviation) or median (with interquartile range (IQR**)**) while categorical variables were expressed as percentage. Independent samples t-test was used to see the difference in the mean values MDA level, protein carbonyl level reduced glutathione levels, total anti-oxidant capacity albumin level uric acid level between breast cancer case groups and control groups. ANOVA was run to see the difference in the mean values of oxidative stress markers (MDA level, protein carbonyl level reduced glutathione levels, total anti-oxidant capacity albumin level uric acid level) among clinical stages. Correlation analysis was used to see the relationship between, oxidant markers and antioxidant markers. Linear regression model was constructed to see association between some socio-demographic and clinical variables with oxidant or antioxidant markers. All the tests were considered significant at *p*-value less than 0.05. Because the distributions of protein carbonyl, total antioxidant capacity, reduced glutathione, albumin and uric acid were not normally distributed, these values were log transformed and used in statistical tests.

4. Result

4.1 Socio-demographic characteristics of study participants

A total of 119 study participants (95 cases and 24 control groups) were included. The median age of the participants was 40 (Range 21-80 years), with the median of age for cases and controls was 40 (21-65) and 30 (Range 23-50 years). The socio-demographic characteristics are displayed in table 1 below. Larger proportion breast cancer patients (cases) were under the age of 45 (67.3%). While larger proportion of the cases were illiterate 47 (49.5%) or attended high school grades 45 (47.4%), participants in the control group were attended high school (41.7%) or higher level education (college diploma or above) (45.8%). The majority of the study participants in both groups were married (cases 78.9% and controls 62.5%), mainly dwelling in the urban areas (76.8% cases) and (100% controls). The mean body mass index in patients with breast cancer (22.23) was similar with the controls (22.17).

Table 1: Socio-demographic characteristics of study participants

Characteristics		Frequency (%)	
		Cases (n=95)	Controls (n=24)
Age group	20- 35	36 (36.8)	14 (58)
	36-45	29 (30.5)	6 (25.2)
	46-55	20 (21.1)	2 (8.4)
	56-65	11 (11.6)	2 (8.4)
BMI group	≤ 18 (underweight)	6 (6.3)	1 (4.2)
	18.5-24.9 (normal weight)	70 (73.7)	15 (75)
	25-29.9(over weight)	19 (20.0)	5 (20.9)
Educational status	Illiterate	47 (49.5)	3 (12.50
	High school /less	45 (47.4)	10 (41.7)
	College / Above	3 (3.2)	11(45.8)
Marital status	Single	11 (11.6)	9(37.5)
	Married	75 (78.9)	15(62.5)
	Widowed	9 (9.5)	0 (0.00)
Region	Addis Ababa	50 (52.6)	23(95.8)
	Oromiya	20 (21.1)	1(4.2)
	Amhara	7 (7.4)	0 (0.00)
	SNNP	12 (12.6)	0 (0.00)
	Tigray	2 (2.1)	0 (0.00)
	Harari	4 (4.3)	0 (0.00)
Residence area	Urban	73 (76.8)	24 (100)
	Rural	22 (23.2)	0 (0.00)

Table 1: Socio-demographic characteristics… (Cont'd)

Characteristics		Frequency (%)	
		Cases (N=95)	Controls (N=24)
Family history of breast cancer	Yes	2 (2.1)	0 (0.00)
	No	93 (97.9)	24 (100)
Family history of ovarian cancer and other cancer	Yes	2 (2.1)	0 (0.00)
	No	93 (97.9)	24 (100)
OCP use	No	79 (83.2)	13 (54.2)
	Yes	16 (17.9)	11(45.2)
Duration of OCP use	1-3 Years	8 (8.4)	6 (25)
	4-6 Years	4 (4.2)	2 (8.3)
	≥ 7 Years	4 (4.2)	0 (0.00)
Menopausal status	Pre-menopausal	53 (55.8)	21 (87.5)
	Post-menopausal	42 (44.2)	3 (12.5)
Age at menarche	≤ 12 Years old	6 (6.3)	2(8.3)
	13-15 Years old	62 (65.3)	19 (79.3)
	16-18 Years old	27 (28.4)	3 (12.5)

Larger proportion of the study participants were premenopausal (55.8% cases and 87.5% controls) with history of OCP use accounted for 74.7% (cases) and 54.2% (controls). The majority of them also had age range at menarche 13-15 years (65.3% cases and 79.3% controls). Premenopausal woman accounted for 55.8 % (cases) and 87.5% (controls) (Table 1).

Table 2 depicts the nutritional habit, alcohol use and physical exercise habits of the study participants. The majority of them had no history of alcohol consumption (80% cases and 87.5% controls). There was no statistical significant difference between cases and control groups in terms of alcohol consumption, physical exercise habits, high fat diet consumption and vegetable and fruit consumption habits ($p > 0.05$).

Table 2: Nutritional habit, alcohol use and physical exercise habits of study participants

Characteristics		Frequency (%)	
		Cases (N=95)	Controls (N=24)
Alcohol Use	None	83 (87.4)	21(87.5)
	1-3 glasses /week	7 (7.4)	3(12.5)
	4-6 glasses / week	5 (5.3)	0 (0.00)
Physical exercise habits	Never	60 (63.2)	15 (62.5)
	Once a week	8 (8.4)	0 (0.00)
	2-3 times a week	20 (21.1)	6 (25)
	4-6 times a week	12 (12.6)	3 (12.5)
High fat diet consumption	Rarely	54 (56.8)	12 (50.00)
	Once a week	8 (8.4)	0 (0.00)
	2-3 times a week	24 (25.3)	10 (41.7)
	4-6 times a week	9 (9.5)	2 (8.3)
Vegetable and fruit consumption	Rarely	33(34.7)	12 (50.00)
	Once a week	5 (5.3)	2(8.3)
	2-3 times a week	48 (50.5)	7 (29.2)
	4-6 times a week	9 (9.5)	3 (12.5)

4.2 Clinico-pathological characteristics of breast cancer patients

Larger proportion of patients had cancer lesion on the left breast 51 (53.7%) compared to right breast cancer 33 (34.7), or bilateral cancer 11 (9.1) and this was statistically significant ($p < 0.001$). Similarly, ductal carcinoma was the dominant form of cancer ($p < 0.001$). In addition, advanced stage disease forms (stages III and IV breast cancer combined) accounted for about 55.8% (table 3)

Table 3: Clinico-pathological characteristics of breast cancer patients

Clinico-pathologic characterstics		Frequency (%)
Tumor size	Less than 2 cm	17 (17.9)
	2-5 cm	38 (40)
	> 5cm	30 (31.6)
	Any size with metastasis to bone ,liver ,lung and brain	10 (10.5)
Histological grade	Poorly differentiated	25 (26.3)
	Moderately differentiated	49 (51.6)
	Well differentiated	21 (22.1)
Site of lesion	Right	33 (34.7)
	Left	51 (53.7)
	Bilateral	11 (9.1)
Type of breast cancer	Ductal	74 (77.9)
	Lobular	16 (16.8)
	Mucinous	5 (5.3)
Stage at time of diagnosis	Stage I	19 (20)
	Stage II	23 (24.2)
	Stage III	25 (26.3)
	Stage IV	28 (29.5)

4.3 Biochemical parameters

4.3.1 Serum oxidant and antioxidant level

The mean malondialdehyde and median protein carbonyl levels in breast cancer patients were (2.44± 1.43) and (38.06 ± 3.29), respectively (table 4). Similarly, the median total antioxidant capacity, reduced glutathione, uric acid and albumin levels in the breast cancer cases were 0.902 (± 0.75), 2.082 (± 3.14), 3.3 (± 1.4) and 3.9 (± 0.3), respectively (table 4). Serum uric acid were significantly higher ($p < 0.05$) compared to the control samples (table 4). Whereas, the levels of TAC, GSH and Albumin were significantly lower than the control groups ($p<0.05$).

Table 4: Serum oxidant and antioxidant levels among breast cancer patients and healthy control samples, Tikur Anbessa teaching hospital, January, 2016.

	Control N=24	Cases (total) N=95	Stage I n=19	Stage II n=23	Stage III n=25	Stage IV n=28
MDA level (µmol /ml) (Mean ± SD)	0.53 ± 0.26 [a]	2.440 ±1.43	1.08 ± 1.37 [de]	1.68 ± 1.3 [ade]	3.501 ± 0.26 [abc]	3.069 ± 1.09 [abc]
Protein carbonyl (nmol/ml) Median (IQR)	17.16 (17.5) [a]	38.06 (3.29)	13.52 (10.02) [cde]	26.8 (15.9) [abde]	46.3 (21.8) [abce]	56.19 (22.5) [abcd]
TAC (mmol Trolox eq./L) (Median + IQR)	1.182 (0.44) [a]	0.902 (0.75)	0.822 (0.46) [ae]	0.92 (0.72) [ad]	0.62 (0.7) [d]	1.212 (0.7) [cd]
GSH level (µM) (Median + IQR)	3.870 (5.01) [a]	2.082 (3.14)	5.15 (4.728) [de]	3.64 (6.23) [de]	1.89 (1.7) [abc]	0.895 (1.6) [abc]
Uric acid (mg/dl) Median (IQR)	2.6 (0.77) [a]	3.3 (1.4)	3.3 (1.4) [b]	3.1 (1.9)[c]	3.3 (1.9)[d]	3.55 (1.1)[e]
Albumin (mg/dl) Median (IQR)	3.950 (0.17) [a]	3.9 (0.3)	3.8(0.5) [acde]	3.9 (0.3) [ab]	3.9 (0.3) [b]	3.9 (0.37) [b]

* Result expressed: mean ± SD, or median + interquartile range

*Data with different alphabetical letters are statistically significant ($p \leq 0.05$)

Independent sample t-test showed that there was a statistically significant difference in MDA and protein carbonyl levels between case and control groups ($p < 0.05$) (table 5) and that the breast cancer patient groups had significantly higher levels of oxidant markers (MDA, and protein carbonyl level).

Table 5: Independent sample t-test

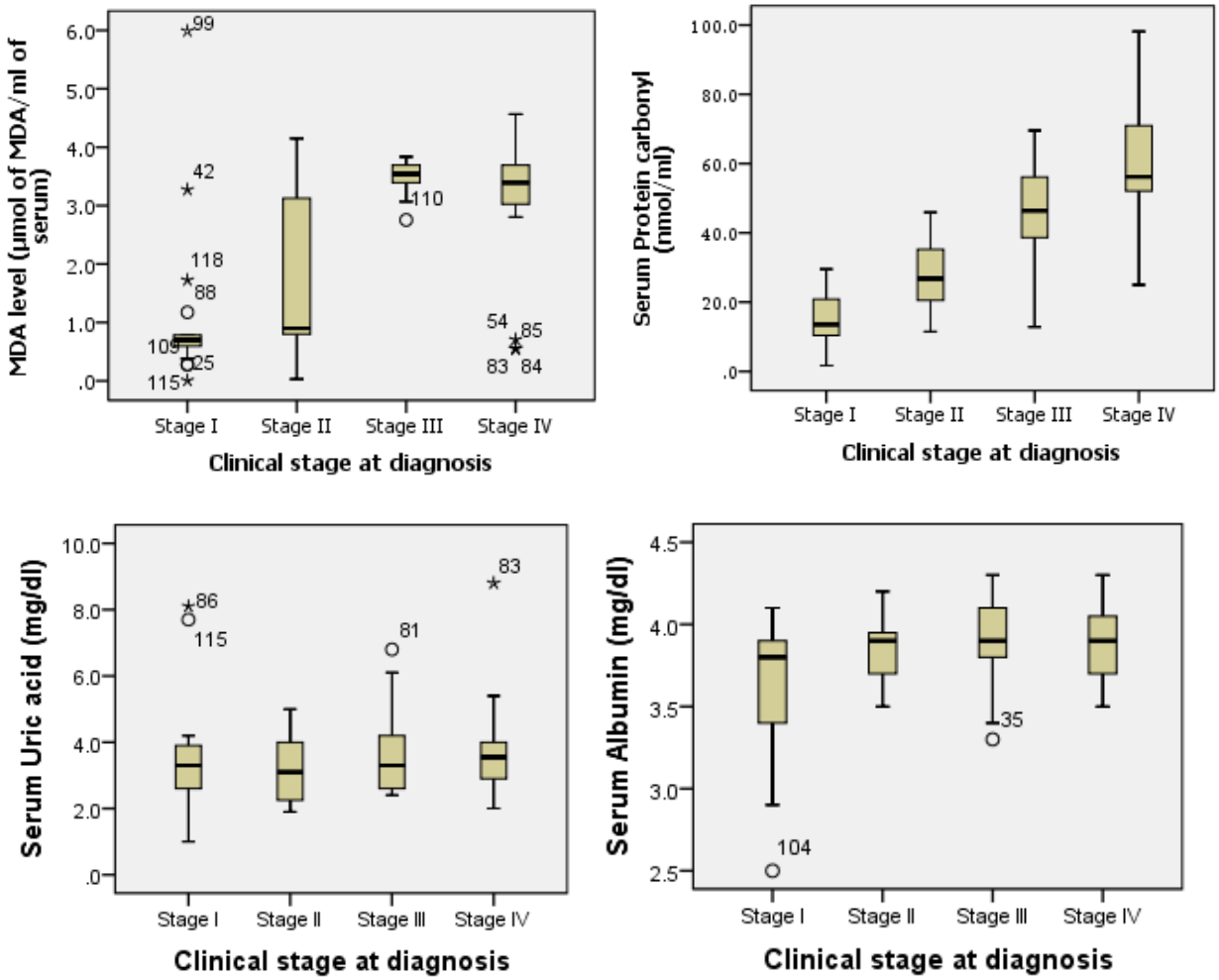
	t	df	P value	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
MDA level (µmol of MDA/ml)	-6.471	117	.000	-1.9117	-2.4968	-1.3266
	-12.190	112.411	.000	-1.9117	-2.2224	-1.6010
Log protein carbonyl (nmol/ml)	-5.756	117	.000	-.40038	-.53814	-.26262
	-5.465	33.449	.000	-.40038	-.54936	-.25140
Log TAC (mmol Trolox eq./l)	2.773	117	.006	.22998	.06571	.39426
	4.897	116.114	.000	.22998	.13696	.32301
Log GSH (µM)	2.900	117	.004	.39020	.12374	.65666
	4.443	85.464	.000	.39020	.21559	.56482
Log Uric acid (mg/dL**)**	-2.509	117	.013	-.08060	-.14422	-.01697
	-3.348	59.175	.001	-.08060	-.12877	-.03243
Log Albumin (mg/dL**)**	2.318	117	.022	.01666	.00242	.03090
	3.873	106.909	.000	.01666	.00813	.02519

Analysis of variance showed statistically significant differences among the different clinical stages in average values of MDA ($p = 0.0001$), protein carbonyl ($p = 0.0001$)), TAC ($p = 0.005$), GSH ($p = 0.0001$), uric acid ($p = 0.043$) and albumin ($p = 0.0001$) (table 6). Tumor size and degree of differentiation of the tumor were also associated with MDA, protein carbonyl, GSH and serum albumin levels ($p < 0.05$) (table 6). To see the trend of clinical characteristics (tumor size, clinical stage, histological grade) on oxidant and antioxidant markers, linear regression analysis was conducted and summarized in table 6.

Table 6: Analysis of Variance in oxidant and antioxidant markers and between clinical Characteristics

		Sum of Squares	Mean Square	F	Sig.
Clinical stage					
MDA level (µmol of MDA	Between Groups	158.888	39.722	42.403	0.000
	Within Groups	106.793	.937		
Log protein carbonyl	Between Groups	8.379	2.095	43.105	0.000
	Within Groups	5.540	.049		
Log TAC	Between Groups	2.011	.503	3.972	0.005
	Within Groups	14.426	.127		
Log GSH	Between Groups	13.206	3.302	12.425	0.000
	Within Groups	30.291	.266		
Log Uric acid	Between Groups	.200	.050	2.548	0.043
	Within Groups	2.238	.020		
Log Albumin	Between Groups	.020	.005	5.613	0.000
	Within Groups	.101	.001		
Tumor size					
MDA level (µmol of MDA/ml of serum)	Between Groups	30.124	10.041	5.573	0.001
	Within Groups	163.955	1.802		
Serum Protein carbonyl (nmol/ml)	Between Groups	9978.996	3326.332	9.018	0.000
	Within Groups	33564.187	368.837		
Serum Reduced Glutathione level (µM)	Between Groups	97.167	32.389	2.821	0.043
	Within Groups	1044.649	11.480		
Serum Albumin (mg/dl)	Between Groups	.740	.247	3.318	0.023
	Within Groups	6.769	.074		
Histological grade					
MDA level (µmol of MDA/ml of serum)	Between Groups	19.370	9.685	5.100	0.008
	Within Groups	174.710	1.899		
Serum Protein carbonyl (nmol/ml)	Between Groups	6479.783	3239.892	8.042	0.001
	Within Groups	37063.400	402.863		
Serum Reduced Glutathione level (µM)	Between Groups	126.732	63.366	5.743	0.004
	Within Groups	1015.085	11.034		

It was observed that there was a significant change in the level of MDA, protein carbonyl, albumin, GSH, and TAC across the clinical stages of breast cancer. Accordingly, for a one unit increase to a higher level clinical stage, there was an increase in MDA level by 0.584 (R^2 = 0.342, p < 0.001), protein carbonyl level by 0.795 (R^2 = 0.0632, p < 0.0001), albumin level by 0.282 (R^2 = 0.079, p = 0.006) and TAC level by 0.248 (R^2 = 0.052, p = 0.015). Thus, as shown in figure 3, these characteristics are increased more among advanced stage patients (stage III and IV) than with early stages (stage I and stage II) breast cancer patients (figure 1). The level of GSH was found to decrease by 0.514 (R^2 = 0.256, p 0.0001) as disease progress to a higher clinical stages. On the other hand, the level of uric acid was insignificantly changing with clinical stage (p = 0.428). Similarly, total antioxidant capacity (TAC) was not statistically associated with tumor size and histological stage (p > 0.05).



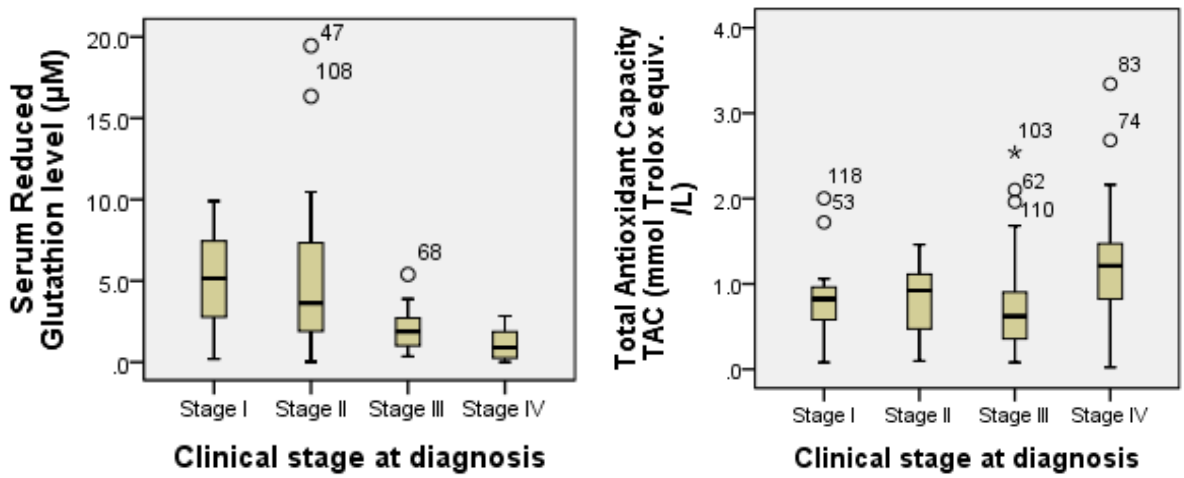


Figure 1: Box plot showing the trend of oxidant and antioxidant levels along breast cancer clinical stages.

Table 7: Linear regression analysis to see the effect of clinical characteristics on serum oxidant and antioxidant levels.

	Serum MDA level		**Serum protein carbonyl level**	
	R^2	p-value	R^2	p-value
Clinical stage	0.342	< 0.005	0.632	< 0.001
Tumor size	0.138	< 0.001	0.215	< 0.001
Histological grade	0.083	< 0.005	0.119	< 0.001

	Serum GSH level		**Serum TAC level**	
	R^2	p-value	R^2	p-value
Clinical stage	0.256	< 0.001	0.052	0.015
Tumor size	0.082	< 0.001		
Histological grade	0.088	< 0.005		

4.3.2 Association Of Nutritional Habit, Alcohol Use And Physical Exercise Habit And Oxidative Stress Markers

In our study, age group, BMI group, nutritional habits, alcohol use and physical exercise among breast case patients were assessed for possible association with serum oxidant and antioxidant markers and displayed in table 8 below and figure 2. There was statistical significant difference in serum albumin level between different age groups ($p = 0.044$) and found to decrease with age ($R2 = 0.052$, $p = 0.026$). Serum TAC and uric acid levels were significantly associated with BMI groups ($p < 0.05$). Similarly, alcohol use habit was also associated with serum protein carbonyl level ($p = 0.011$), while, physical exercise and high fat diet were significantly associated with GSH ($p = 0.002$) and TAC ($p = 0.011$), respectively.

Table 8: Analysis of variance of some socio-demographic factors with oxidant and Antioxidant markers

ANOVA						
Age Group		**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Serum Albumin (mg/dl)	Between Groups	.765	4	.191	2.551	0.044
	Within Groups	6.744	90	.075		
BMI Group						
Total Antioxidant Capacity TAC (mmol Trolox equiv./L)	Between Groups	6.111	3	2.037	6.133	0.001
	Within Groups	30.223	91	.332		
Serum Uric acid (mg/dl)	Between Groups	39.350	3	13.117	9.639	0.000
	Within Groups	123.830	91	1.361		
Alcohol use						
Serum Protein carbonyl (nmol/ml)	Between Groups	5787.188	4	1446.797	3.449	0.011
	Within Groups	37755.995	90	419.511		
Physical exercise habit						
Serum Reduced Glutathione level (µM)	Between Groups	211.416	5	42.283	4.045	0.002
	Within Groups	930.401	89	10.454		
High fat diet						
Total Antioxidant Capacity TAC (mmol Trolox equiv./L)	Between Groups	4.846	4	1.211	3.463	0.011
	Within Groups	31.487	90	.350		

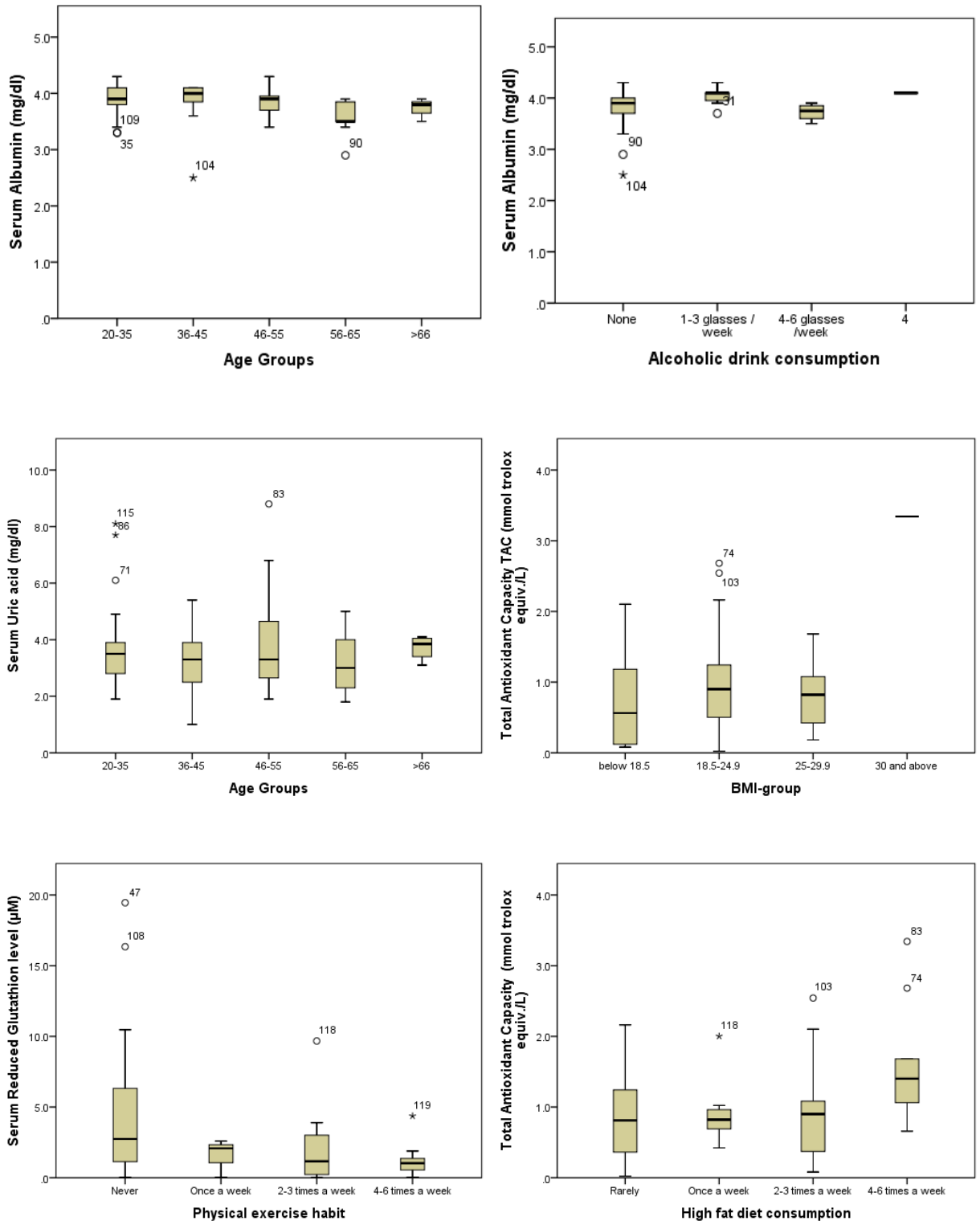


Figure 2: relationship between nutritional habit, alcohol use and physical exercise habit against biomarkers of oxidative stress

4.3.3 Correlations analysis between oxidant and antioxidant markers

To study, the relationship between oxidant and antioxidant markers, *Pearson* correlation analysis (Table 9) followed by linear regression modeling (figure 5) was conducted. It was shown that the oxidant markers (serum levels of MDA and protein carbonyl) were correlated with the antioxidant markers (TAC, glutathione (GSH) and Albumin) with significant association ($p < 0.001$). A positive correlation was also observed between level of MDA and protein carbonyl with significant association in patients with breast carcinoma ($p < 0.001$) (Table 9). The regression analysis (figure 5 below) indicated that, as MDA level increases, the levels of protein carbonyl (R^2 0.132, $p < 0.0001$), and albumin ($R^2 = 0.087$, $p = 0.004$) were found to increase, but the levels of GSH got decreased (R^2 0.089, $p = 0.003$) (figure 5). Similarly, an increase in serum protein carbonyl level was associated with an increase in the levels of albumin ($R^2 = 0.075$, $p = 0.007$) and a decrease of GSH level ($R^2 = 0.178$, $p < 0.0001$) (Figure 5).

Table 9: Correlation analysis between oxidant and antioxidant markers

Correlations

		MDA level (µmol of MDA/ml of serum)	Serum Protein carbonyl (nmol/ml)	Serum Albumin (mg/dl)	Total Antioxidant Capacity TAC (mmol Trolox equiv./L)	Serum Reduced Glutathione level (µM)
MDA level (µmol of MDA/ml of serum)	Pearson Correlation	1	.431**	0.296**	-.024	-.386**
	Sig. (2-tailed)		.000	0.004	.817	.000
	N	95	95	95	95	95
Serum Protein carbonyl (nmol/ml)	Pearson Correlation	.431**	1	0.223*	.274**	-.457**
	Sig. (2-tailed)	.000		0.030	.007	.000
	N	95	95	95	95	95

**. Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

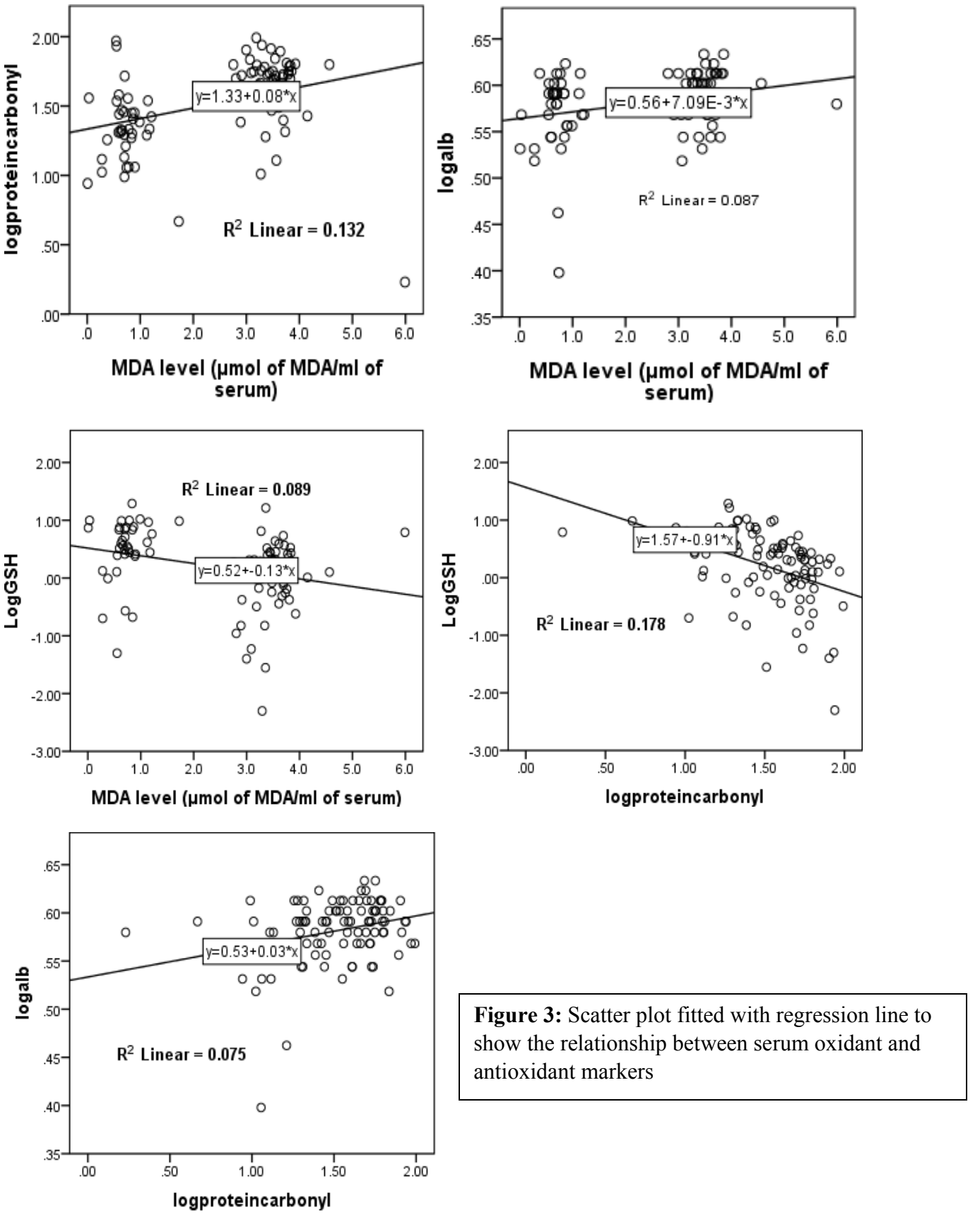


Figure 3: Scatter plot fitted with regression line to show the relationship between serum oxidant and antioxidant markers

5. Discussion

In order to develop an effective strategy to prevent and treat disorders including cancer, an accurate evaluation of ROS and antioxidants is of a critical importance. The understanding of causes and progression of breast cancer contributes to the development of proper diagnosis, prevention, or treatment approach. In the etiology of breast cancer, radiation, high fat diet, chemical agents, drugs, chromosomal abnormalities have been incriminated along with oxidative stress. The damage due to oxidative stress can be to proteins, DNA, (causing mutations and oncogenesis) or lipids causing membrane damage (Barrera 2012).

In this study, serum biomarkers of oxidative stress such as malondialdehyde (as indicator of lipid peroxidation), protein carbonyl levels (as marker of protein oxidation) and levels of reduced glutathione (GSH), total antioxidant capacity (TAC), uric acid and albumin (as indicators of body's endogenous antioxidant ability) were studied among breast cancer patients and healthy controls. The present study result demonstrated that there were statistically significantly higher serum MDA and protein carbonyl levels in breast cancer group than in control group (p-value < 0.05) while the levels of TAC, GSH, albumin and uric acid were decreased. Patients with the highest mean serum MDA and protein carbonyl levels were those with advanced breast cancer (stages III and IV) as compared to early stage diseases (stages I and II) (p-value < 0.05). Among the case groups, as breast cancer progress to a higher stages, the levels of MDA, protein carbonyl, TAC, albumin were found to increase ($p < 0.05$).

As reviewed by Södergren, (2000), lipid peroxidation is one of the most extensively investigated free radical–induced process, polyunsaturated fatty acids containing two or more double bonds, are particularly susceptible to peroxidation, and once the process is initiated, it proceeds as a free radical–mediated chain reaction involving initiation, propagation, and termination . According to the studies conducted by the following scholars Karihtala and Puistola, (2011) ; Karihtala *et al.,* (2011); Pande *et al.,*(2011); Vera-Ramirez *et al.*,(2011); Pande *et al.,*(2012) levels of lipid peroxidation markers MDA and 4-HNE, are elevated in breast cancer tissue and in the serum and urine of breast cancer patients when compared with healthy controls .

In this study, patients with breast cancer exhibited higher levels of MDA in their serum compared to the control group. According to studies done by Ray *et al*., (2000), Gonenc *et al*., (2001); Yeh *et al.,* (2005), Sheeba *et al*., (2010), they also showed consistent results that breast cancer patients had higher concentration of MDA than the control group. The reason for this elevated level of serum MDA in breast cancer patients may be due to defective antioxidant system which is associated with the oxidative stress that leads to loss of polyunsaturated fatty acid in membranes. This defect affect membrane integrity this in turn leads to the accumulation of lipid peroxides in the circulation of those patients this proposed hypothesis is also supported by Gonenc *et al*., (2001).

Our study result demonstrated that patients in stage III and IV had the higher levels of MDA in their serum as compared to stages I and II ($p < 0.0001$). Ray *et al*, (2000) also observed that serum MDA concentration had significantly increased in stage II and III of the disease. These results might demonstrate the increased free radical activity with increasing severity of breast cancer. In contrary to our finding, Gonenc *et al*., (2005) reported a decreased serum MDA levels in which advanced breast cancer patients in comparison to benign group (stage I and II) ($p < 0.05$). In contrast, Alagöl and his collaborates (2010) reported lower MDA levels had also been demonstrated in patients with advanced breast cancer (III and IV). According to Alagöl *et al*., (2010) decreased lipid peroxidation products including MDA give transformed cells a selective growth advantage.

As shown in table 6, serum MDA level was also significantly associated with degree of tumor differentiation, such that., higher level of MDA was observed in patients with moderately (grade II) and well differentiated (grade I) tumor compared to poorly differentiated (grade III) tumor of breast cancer. In poorly differentiated tumor, nuclei are spread all over the tumor area and found irregular in shape, which means formation of new cells and nuclei occurred. Since older cells have increased ROS than new cells, hence due to appearance of new cells, decreased level of ROS may be observed in poorly differentiated cancer, as a result of new electron transport chain machinery. Moreover, new cells emanate with larger amount of antioxidant enzymes and molecules than older cells do. These antioxidants might be able to compensate for lost ones to trap the reactive species (Leutner *et al*., 2001).

Plasma/serum protein carbonyl levels were significantly higher in breast cancer patients than in healthy women (Rossner *et al.,* 2007; Tesarova *et al*., 2007) suggesting that enhanced protein carbonyl concentrations are associated with breast cancer (Rossner *et al.,* 2007).

In agreement with previous studies by Rossner *et al., (*2007); Tesarova *et al*., (2007), Badid *et al*., (2012), Panis *et al*., (2012),our study also showed a significantly increased level of protein carbonyl in breast cancer patients as compared to normal controls ($p < 0.0001$). This high levels of protein carbonyl in the serum of breast cancer patients as compared to health controls indicates that in our finding breast cancer has been associated with sever systemic oxidative damage to plasma proteins.

In this study, the serum levels of carbonyl proteins were demonstrated to be associated with the clinical stages of breast cancer ($p < 0.0001$). Breast cancer patients with the highest log mean serum protein carbonyl levels were those with advanced stages (stage III and IV) of breast cancer when compared with early stages (stage I and II) ($p < 0.05$). This might indicate that the carbonyl level was positively associated with the progression of the disease.

As with the elevated serum MDA level, we also found higher protein carbonyl levels in our breast cancer patients with well and moderately differentiated tumor compared to the poorly differentiate tumor. This might be attributed to hypothesis that well and moderately differentiated tumors have been proposed to be dominated by older cells with increased ROS production than newer tumor cells (Leutner *et al*., 2001). In this study, we have also found that serum protein carbonyl level was associated with alcohol consumption. Report by Türkoğlu *et al.,* (2000), showed that ethanol metabolism can generate free radicals. However, components of some alcoholic beverages, such as resveratrol in red wine, have also been shown to possess antioxidant properties.

TAC estimates peroxyl- scavenging capacity of the extracellular antioxidant system, comprised of protein thiol groups (52.9%), uric acid (33.1%), vitamin C (4.7%), bilirubin (2.4%), vitamin E (1.7%) and unidentified antioxidants (5.2%) (Bartosz G. 2010).

The present study depicted decreased serum total antioxidant capacity (TAC) in breast cancer patient in comparison to the controls ($p < 0.05$). This finding was also in agreement with the study done by Sener *et al*., (2007), who showed that TAC level was decreased significantly in patient groups as compared to health controls ($p < 0.05$). The reduction in TAC in breast cancer patients could reflect consumption of endogenous antioxidants by enhanced reactive oxygen species generation by the disease process or insufficient dietary intake of exogenous antioxidants might have also contributed.

Although the median of TAC was decreased in breast cancer patient compared to controls, significantly higher TAC was observed in stage IV patients compared to stages II and stage III ($p = 0.018$). This might be described by the fact that the antioxidant system responds oxidative stress constantly and prevents damages by scavenging ROS, but this balance is restricted to healthy participants in our study. But as the breast cancer progresses to advanced stages, increase markedly without even reducing of antioxidant capacity. Consequently, increased ROS production may have other more influence on the effect for breast cancer patients than reducing antioxidant markers as showed total antioxidant capacity. Furthermore, the levels of serum total antioxidants were negatively correlated with intake of high-fat diet. This can be explain based on report by Vieira and his colleagues (2011),they showed high intake of dietary fat directly enhanced ROS overproduction which increased lipid peroxidation and decreased antioxidant activities.

Reduced glutathione play multidirectional role in cellular processes including cell growth, apoptosis, defense against oxidative stress as well as in chemo resistance and progression of cancer. According to Traverse *et al*., (2013) a decrease in GSH level in general is an indication of oxidative stress but on other hand a rise in its level supports metastatic invasions of cancer. In patients with breast cancer, GSH was found to be elevated according to studies done by Ghalia and Fouad, (2000) and Javed *et al* .,(2015) ,while it was reduced in others studies by Mahajan *et al*.,(2013), Amin *et al .,* (2012) and Kumaraguruparan *et al* .,(2002). We have found significantly lower values of serum GSH in breast cancer patients as compared to levels in controls ($p < 0.05$).

Our finding is also in line with previous study report by Hefny *et al*. (2009). The decreased GSH concentration seen in the circulation of breast cancer patients can be explained by a decrease in glutathione synthesis and/or increased consumption to remove peroxides. This proposed mechanism is supported by Aggarwal *et al*., (2006). In addition, we also found significant decrease in the mean levels of GSH in stage IV (compared to stage I ($p < 0.0001$), stage II ($p < 0.0001$) as well as stage III disease ($p < 0.015$) indicating that scavenging ability of glutathione was greatly decreased in advanced stages as compared to early stage or locally advanced stage of the disease. Thus the deceased GSH levels in different stages of breast cancer coincide with the enhanced lipid peroxidation and protein oxidation observed in corresponding stages of breast cancer patients. The significantly decreased GSH levels in breast cancer patients may be attributed to increased utilization to scavenge lipid peroxides as well as sequestration by tumor cells.

The present study also demonstrated increased serum GSH levels in breast cancer patients with higher physical activity (≤ 2-3 times a week) was associated with decreased serum protein carbonyl levels ($p<0.001$). According to Urso and Clarkson (2003), they explained that, higher (strenuous) physical exercises can produce an imbalance between ROS and antioxidants and thus lead to oxidative stress.

Uric acid, as antioxidant, may play a crucial role in cancer etiology by preventing the formation of oxygen radicals, thereby protecting against carcinogenesis (Ames *et al*., 1981; Peden *et al*., 1990). Our study demonstrated decreased serum uric acid levels in breast cancer patients as compared to healthy controls ($p < 0.05$), supporting the depressed antioxidant and protective effect of uric acid against oxidative stress in breast cancer patients. This reduction in serum uric acid may also have contributed to the lower level of TAC observed in these breast cancer patients. The reduction in the levels of uric acid in patients could be attributed to the malignant process resulting from the reduced nucleic acid turnover in the proliferating diseased tissues of those patients. In the study conducted by Bhargava *et al.*, (2015), serum uric acid levels increased in breast cancer patients compared to healthy control subjects and this is similar to reported by Veni *et al.*, (2010) study that showed that uric acid may be a protective agent due its function as antioxidant although, raised serum uric acid is risk factor for incidence and mortality

in breast cancer. According to the finding of this study, a significant rise in uric acid level in untreated women of breast cancer patients, which may be due to high oxidative stress. Contrasting to these findings, a study conducted by Omar *et al.*, (2011), there was a significant decrease in uric acid level observed in breast cancer patients on chemotherapy, levels were significantly lower in metastatic cancer compared with non-metastatic cancer patients. This decrease in serum uric acid might have also contributed to the lower level of total antioxidant capacity of those patients and due to the effect of the chemotherapy.

According to the finding by Sautin and Johnson (2008), uric acid may appears to make a significant contribution to serum antioxidant capacity it has been found to stimulate granulocyte adherence to the endothelium, and peroxide and superoxide free radical liberation. Uric acid may have a deleterious effect on the endothelium through leukocyte activation and, interestingly, a consistent relationship and circulating inflammatory markers.

A cohort study done by Strasak and this colleagues (2007) to investigate the relations of serum uric acid levels to subsequent cancer mortality, high level of serum uric acid was associated with increased risk of total cancer mortality. The study also indicated that serum uric acid levels were positively related to deaths from malignant neoplasms of breast. On the other hand, in our study the level of serum uric acid was not significantly associated with the various clinical characteristics (clinical stages, tumor size and histological grades) of breast cancer patients which may be due to high oxidative stress which may lead to the generation of free radicals was reduced by uric acid.

In our study of uric acid and albumin levels, both serum uric acid and albumin levels cancer patients were significantly higher than those of controls ($P < 0.05$). But uric acid levels in breast did not differ significantly between controls and cases .This finding was in contrary with study by Gonenc *et al*., 2001, they demonstrated that albumin levels did not differ significantly between controls and cases ($P > 0{\cdot}05$), but uric acid levels in breast cancer patients were significantly higher than those of controls ($P < 0.05$).

According to a literature by Halliwell (1988), albumin concentrations were found enhanced in sites of inflammation where the protein exerts its multiple antioxidant properties and high concentrations of circulating albumin indicate that albumin might be able to scavenge some hydroxyl radicals produced from iron's reaction with H_2O_2. In our study, a significant decrease in serum albumin levels in breast cancer was depicted as compared to healthy controls ($p < 0.05$). This finding is in line with previous study done by Gonenc *et al*., (2007). The reason for this decreased serum level of albumin may probably due to the decrease in the levels of antioxidants capacity and also a structural modification of albumin induced by free radicals attack impaired the antioxidative properties of albumin. Furthermore, Koc *et al*., (2003) explained that albumin is a negative acute phase protein synthesized by liver whose levels fall in response to infection, injury and neoplasia.

Furthermore, in this study the levels of serum albumin of breast cancer patients were positively correlated with serum MDA and protein carbonyl, this finding can be explained albumin possesses antioxidant properties including binding copper tightly and iron weakly, scavenging free radicals, and providing thiol group. ROS induced damage to protein and lipid leads to increase in oxidation of human serum albumin. This raised level of albumin may due to availability of oxidants and to be oxidized.

In this study, a negative correlation was observed between serum levels of GSH and serum levels MDA as well as protein carbonyl. This observation demonstrates that the increased levels of both of these oxidants are accompanied by a decrease in GSH in those patients. In the breast cancer patients, since decreased level of serum GSH, indicating inadequate antioxidant protection, resulting in significantly elevated level of lipid peroxidation and protein oxidation. High consumption of enzymatic and non-enzymatic antioxidant in cells would naturally lead to low extracellular levels of antioxidants. According to the study done by Ramírez-Expósito *et al*., (2014), oxidatively induced damage, caused by decreased capacity for ROS removal, is associated with suppressed antioxidant action of reduced glutathione.

6. Conclusion and recommendation

6.1 Conclusion

- The present study demonstrated oxidative stress in breast cancer patients, indicated by increased oxidative parameters (MDA as well as protein carbonyl level) and decreased antioxidant parameters (TAC, GSH, uric acid and albumin levels) when compared to healthy controls.

- Patients with breast cancer suffer a high degree of oxidative stress which associated with decrease antioxidant system level and significant degree of lipid peroxidation and protein oxidation.

- Clinical stages of breast cancer were associated with oxidative stress that increase of oxidative stress markers in patients as clinical stages increases. Moreover, the study also supports the possibility of involvement of oxidative stress in progression of the disease.

- The study also found imbalance oxidant and antioxidant status among clinical stages and other clinical characterstics (including tumor size, and histological grades) and which may be the key role in the pathogenesis and progression of breast cancer.

- The oxidative and antioxidant markers showed a negative correlation in patients with breast cancer indicates that a higher oxidants and inefficient antioxidant defense system may play a significant role in the increased oxidative stress found in those breast cancer patients.

6.2 Recommendations

- This study is cross sectional and more studies need be done in larger participants using additional oxidative stress markers including oxidatively damaged DNA metabolites and important enzymatic antioxidants.

- Chemotherapy and radiotherapy induce the production of free radicals; and thus, assessment of oxidant /antioxidant status of breast cancer patients on treatment is recommended.

- Controlled studies needs to be conducted to see the impact of antioxidant supplementation in breast cancer patients in an attempt to decrease the effect of oxidative stress.

7. Limitations and strength of the study

7.1 Limitations of the study

Although this study revealed pertinent information with respect to oxidative stress in breast cancer patients, the sample size was small and may not show the precise picture of the problem. The cost of reagents and supplies were high so that we have limited ourselves to the 119 study participants. Even under this condition, power analysis showed that the study has 79.27% power to detect a significant difference at the significance level of 0.05 (95% confidence level). In addition we did not investigate the impact of treatment, for example surgery, on oxidative stress. The impact of oxidative stress on the level and activities of enzymatic antioxidants as well as on DNA damage in breast cancer patients has not been determined in this study

7.2 Strength of the study

However, relatively comprehensive standard test methods (such as malondialdehyde (MDA) protein carbonyl, reduced glutathione, total antioxidant capacity, uric acid and albumin) have been used to assess the status of oxidative stress in breast cancer patients in Ethiopia. Moreover, the study involved healthy control samples to clearly show the contrast in oxidative stress observed in patients

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Annexes

Annex 1: Questionnaire

Please answer every question in the questionnaire by marking "X" in the space or filling the necessary information.

Code NO_____

Part 1: **Socio-Demographic characteristics**

1.1. Age_____

1.2. Region _____

1.3. Residence area: Rural_____Urban_____

1.4. Education level: Illiterate _____High School or less_____college or above_____

1.5. Marital status: Single_____Married_____Widowed_____

1.6. Height_____ Weight_____ BMI_____

Part 2: Clinico-pathological characteristics

2.1 Family history

2.1.1 Has your mother ever had breast cancer diagnosed?

☐ No ☐ Don't know

☐ Yes, If **Yes**, how old was she when the cancer was first diagnosed? ☐☐ Years

2.1.2 How many sisters do you have? ☐☐ Sisters

2.1.3 Have any of your sisters ever had breast cancer diagnosed?

☐ No/No sisters ☐ Don't know

☐ Yes- If **Yes**, how old was they when the cancer was first diagnosed?

1st sister ☐☐ years 2nd sister ☐☐ years

2.1.4 Have you ever had breast cancer diagnosed?

☐ No

☐ Yes- If **Yes**, how old was you when the cancer was first diagnosed? ☐☐ Years

2.1.5 Have you ever had any family history of ovarian cancer? ☐ Yes ☐ No

2.1.3. Have you ever had Family history of other type of cancer? ☐ Yes ☐ No

Please specify:

2.3 Alcohol consumption

About how much wine or beer (or other alcoholic drink) do you drink on average *each week*?

Wine /Beer (or other alcoholic drink) **(*glasses per week*)**

- ☐ None
- ☐ Less than 1
- ☐ 1-3
- ☐ 4-6
- ☐ 7-10
- ☐ 11-15
- ☐ 16-20
- ☐ 21+

2.4 Exercise activities

How often do you do any *(can be strenuous)* exercise?

- ☐ Rarely/never
- ☐ Less than once a week
- ☐ Once a week every day
- ☐ 2-3 times a week
- ☐ 4-6 times a week

2.5 Oral contraceptive use ☐ Yes ☐ No

2.5.1 About how old were you when you *first went on* the pill? ☐☐ Years

2.5 .2 about how old were you when you *last came off* the pill? ☐☐ Years

2. 6 Reproductive history

2. Age at menarche ☐☐ Years

2. Menstrual status: Pre ☐ Post ☐

2. Age at menopause ☐☐ Years

2.7 Nutritional habits

How often do you do ***consume?***

A) High fat diets (including Animal fat ,Dairy products include butter ,oils ,high-fat meat)

- ☐ Rarely/never
- ☐ Less than once a week
- ☐ Once a week every day
- ☐ 2-3 times a week

B) Vegetables and fruits (enriched in vitamins like vit.D, C)

- ☐ Rarely/never
- ☐ Less than once a week
- ☐ Once a week every day
- ☐ 2-3 times a week
- ☐ 4-6 times

2.8 Are you now being treated for?

- High blood pressure *(hypertension)* ☐ Yes ☐ No
- Heart disease ☐ Yes ☐ No
- Diabetes ☐ Yes ☐ No
- High blood cholesterol ☐ Yes ☐ No
- Liver diseases or injury ☐ Yes ☐ No
- Kidney diseases or injury ☐ Yes ☐ No

2.8.1 Are you **now** being treated for any other *serious* illness? ☐ Yes ☐ No

Please describe this illness

2.9 Exposure to high wave radiation in your working or living environment

☐ No ☐ Yes

Please describe

2.10 **clinical Stage of the breast cancer** at diagnosis: I ☐ II ☐ III ☐ IV ☐

THANK YOU FOR YOUR PARTICIPATION

Annex II: Questionnaire Amharic version

መጠይቅ

እባክዎን በመጠይቅ ላይ ያሉትን ጥያቄዎች "X" በማድረግ ወይም አስፈላጊዉን መረጃ በክፍት ቦታዉ ላይ ይሙሉ::

ኮድ ቁጥር ----------

ክፍል -1 ማህበረሰባዊ -ህዝባዊ ባህሪያት ጥያቄዎች

1.1 እድሜ ----------

1.2 ክልል ----------

1.3 መኖሪያ ስፍራ ገጠር ---------- ከተማ ----------

1.4 የትምህርት ደረጃ ያልተማረ---------- ሁለተኛ ደረጃ ና ከዛ በታች -------- ኮሌጅና ከዛ በላይ ------

1.5 የጋብቻ ሁኔታ ያላገባች ----------ያገባች ----------

1.6 ቁመት ----------ክብደት----------BMI----------

ክፍል -2 ክሊኒካዊ እና ከጤና እክል ጋር የተገናኙ ባህሪያት ጥያቄዎች

2.1 የቤተሰብ መረጃ

2.1.1 በቤተሰብ ዉስጥ እናትሽ የጡት ካንሰር ምርመራ አድርጋ ታዉቃለች

☐ ምርመራ አድርጋ አታዉቅም ☐ ህክምና አድርጋ ታዉቃለች

☐ ምርመራ ካደረገች ፣ እድሜዋ ምን ያህል ነበር የጡት ካንሰር ምርመራ ወቅት ☐☐ አመት

2.1.2 በቤተሰብ ዉስጥ ምን እህቶች አሉሽ ☐☐ እህቶች

2.1.3 ከእህቶች ዉስጥ የጡት ካንሰር ምርመራ አድርጋ/አድርገዉ የሚዉቁ አሉ

☐ ምርመራ አድርጋ/አድርገዉ አታዉቅም/ቁም ☐ አድርጋ/አድርገዉ ታዉቃለችም/አያዉቁም

☐ ምርመራ ካደረጉ፣ እድሜዋ ምን ያህል ነበር የጡት ካንሰር ምርመራ ወቅት ☐☐ አመት

2.1.4 እርሶ ከዚህ በፊት የጡት ካንሰር ህክምና አድርገዉ ያዉቃሉ

☐ ምርመራ አድርጌ አላዉቅም

☐ ምርመራ አድርጌ ነበር፣ እድሜዎ ምን ያህል ነበር በጡት ካንሰር ምርመራ ወቅት ☐☐ አመት

2.1.5 በቤተሰብ ዉስጥ የኦቫሪያን ካንሰር ታሪክ አለ ☐ አለ ☐ የለም

2.1.6 በቤተሰብ ዉስጥ ሌላ የካንሰር አይነት ታሪክ አለ ☐ አለ ☐ የለም

እባክዎን ስለ ካንሰሩ አይነት ይግለጹ

2.2 የአልኮል መጠጥ

ምን ያህል ወይን ወይም ቢራ (ሌሎች የአልኮል መጠጦች) ብርጭቆ በአንድ ሳምንት ዉስጥ ይጠጣሉ

ወይም ቢራ (ሌሎች የአልኮል መጠጦች) ብርጭቆ / በሳምንት

- ☐ ምንም አልኮል አልጠቀምም
- ☐ ከ1 ያነሰ
- ☐ 1-3
- ☐ 4-6
- ☐ 7-10
- ☐ 11-15
- ☐ 16-20
- ☐ 21 በላይ

2.4 የሰዉነት አካላዊ እንቅስቃሴ

የሰዉነት አካላዊ እንቅስቃሴ ምን ያህል ጊዜ ያዘወትራሉ

- ☐ ምንም እንቅስቃሴ አላደርግም
- ☐ ከ 1 ያነሰ ሳምንት
- ☐ በሳምንት 1 ቀን
- ☐ 2-3 ጊዜ በሳምንት
- ☐ 4-6 ጊዜ በሳምንት

2. 5 በአፍ የሚወሰደ የወሊድ መቆጣጠሪያ እንክል ይጠቀሙ ነበር ☐ አዎ ☐ አልጠቀምም

2.5.1 የወሊድ መቆጣጠሪያ እንክል ለመጀመሪያ ጊዜ ሲጠቀሙ እድሜዎ ምን ያህል ነበር ☐☐ አመት

2.5.2 የወሊድ መቆጣጠሪያ እንክል ለመጨረሻ ጊዜ ሲጠቀሙ እድሜዎ ምን ያህል ነበር ☐☐ አመት

2.6 ስነ-ተዋልዶ

2.6.1 የወር አበባ ለመጀመሪያ ጊዜ ያየሸበት እድሜ ☐☐ አመት

2.6.2 የወር አበባ እያየዉ ነዉን ☐ አዎ ☐ አይደለም

2.6.3 አይደለም ከሆነ፣ የወር አበባ ማየት ያቆሙበት እድሜ ☐☐ አመት

2.7 የአመጋገብ ልምድ

ሀ) ብዙ ቅባት የበዛባቸዉን ምግቦች (የእንስሳት ተዋጽኦዎችን ፣የወተት ተዋጽኦዎችን እንደ ቅቤ፣ዘይት የመሳሰሉትን ምን ያህል ጊዜ ያዘወትራሉ

- ☐ አላዘወትርም/አልመገብም
- ☐ ከ1 ያነሰ በሳምንት ዉስጥ
- ☐ 1 ጊዜ በሳምንት ዉስጥ
- ☐ 2-3 ጊዜ በሳምንት ዉስጥ
- ☐ 4-6 ጊዜ በሳምንት ዉስጥ

ለ) አትክልት እና ፍራፍሬ (በ ቫይታሚን የበለጸጉ እንደ ቫይታሚን ዲ፣ሲ ወዘተ) የመሳሰሉትን ምን ያህል ጊዜ ያዘወትራሉ

☐ አላዘወትርም/አልመገብም ☐ 1 ጊዜ በሳምንት ዉስጥ

☐ ከ1 ጊዜ ያነሰ በሳምንት ዉስጥ ☐ 4-6 ጊዜ በሳምንት ዉስጥ

☐ 2-3 ጊዜ በሳምንት ዉስጥ

2.8 በአሁን ወቅት ከዚህ በታች የተዘረዘሩትን የጤና እክሎች ምርመራ እየተከታተሉ ነዉ

- የደም ግፊት ☐ አዎ ☐ አይደለም
- የልብ በሽታ ☐ አዎ ☐ አይደለም
- የስኳር በሽታ ☐ አዎ ☐ አይደለም
- የደም ኮሌስትሮል ☐ አዎ ☐ አይደለም
- የጉበት በሽታ/ጉዳት ☐ አዎ ☐ አይደለም
- የኩላሊት በሽታ/ጉዳት ☐ አዎ ☐ አይደለም

2.8.1 በአሁን ወቅት ከዚህ በላይ የተዘረዘሩትን ዉጪ የሆኑ ከፍተኛ የጤና እክሎች ምርመራ እየተከታተሉ ነዉ

☐ አዎ ☐ አይደለም

ከሆኑ እባክዎን ስለ ህመሞ ይግለጹ:-

2.9 በሚሰሩበት መስሪያ ቤት ወይም (ፋብሪካ) እና መኖሪያ አካባቢዎ በሀይለኛ ና ጎጂ ጨረር ምንጮች ይጋለጣሉ

☐ አዎ ☐ አልጋለጥም

ከሆኑ እባክዎን ይግለጹ :-

2.10 የጡት ካንሰሩ ክሊኒካል ደረጃ በምርመራዉ ጊዜ : I ☐ II ☐ III ☐ IV ☐

Annex III: information sheet

Introduction

I am Yimenashu Mamo, from Addis Ababa University Graduate Studies, Faculty of Medicine, who is conducting my study, on Investigation of serum biomarkers of oxidative stress among various clinical stages of breast cancer patients in Tikur Ambesa Hospital, Addis Ababa Ethiopia for my partial fulfillment of the requirements for the degree of Masters in Medical Biochemistry.

Objective of the study

The overall objective is Investigation of oxidative stress among various clinical stages (stage II, III and VI) of breast cancer patients

Possible risks

This study has no health risk except minimum pain associated with blood with drawl procedure. This is just the result of the routine work which experienced by all the individuals that will involve in the research or the assessment of oxidative stress by experienced health practitioner.

Benefits

Being the member of the research will give you the following benefits from this research; you will have the chance to look the status of oxidant –antioxidant and hematological profiles and may take measure like need to visit clinic for further test to control before worse. You also get necessary advice regarding oxidative stress and its health effects. In addition to this the information you provide is helpful for the health program planners in the Addis Ababa university medical school to improve future prevention of oxidative stress along with breast cancer patient treatment.

Confidentiality

The information you provide is confidential and will only be used for the objective mentioned above. You have the right to refuse participation or quit participation at any point of the interview process if you are not comfortable.

Right to get information

Ethical clearance will be obtained from Addis Ababa University College of Health Sciences Department of Biochemistry. A letter of cooperation will be written from department authorities. The main objective of this committee is to protect the participant from any risk and discomfort which may result due to the procedure of the study.

Annex IV: Patient information a Amharic version

አዲስ አበባ ዩንቨርሲቲ

የሕክምና ፋካልቲ

ባዮኬሚስትሪ ት/ክፍል

የጥናት ተሳታፊዎች የመረጃና የስምምነት ቅፅ

የጥናቱ ርዕስ፡- በጥቁር አንበሳ ስፔሻላይዝድ የመማሪያ ሆስፒታል ዉስጥ በሚገኙ በተለያዩ ደረጃ ላይ ለሚገኙ ጡት ካንሰር ህሙማን የሚከሰተዉን የ (***oxidative stress***") መጠን ለመለካት::

የጥናቱ ባለቤት፡ ይመናሹ ማሞ

አድራሻ፡- ሞባይል +251 912456166

ኢ-ሜይል - yimenashumamo@gmail.com

ክፍል አንድ ፡- የጥናቱ ተሳታፊዎች የመረጃ ቅፅ

መግቢያ ፡- በዚህ ጥናታዊ ዕሁፍ ይ እንዲሳተፉ እየተጋበዙ ነው:: በጥናቱ ላይ ለመሳተፈ ከመወሰኔ በፊት ጥናቱ ለምን እንደሚካሄድና ምን ምን ዓይነት ነገሮች እንደሚያስፈልጎት ማወቅ ነው:: ስለዚህ ጥቂት ጊዜ ይወስዱና የሚከተለውን ስለ ጥናቱ በተመለከተ መረጃ ይመልከቱ አስፈላጊ ከሆነም ከሌሎች ሰዎች ጋር ይወያዩ:: ማንኛውም ግልጽ ያልሆነ ነገር ካለ ወይም ተጨማሪ መረጃ ከፈለጉ የጥናቱ ባለቤት መጠየቅ ይችላሉ::

የጥናቱ አላማ፡- የዚህ የጥናት ዋና አላማ በጥቁር አንበሳ ስፔሻላይዝድ የመማሪያ ሆስፒታል ዉስጥ በሚገኙ በተለያዩ ደረጃ ላይ ለሚገኙ ጡት ካንሰር ህሙማን የሚከሰተዉን የ (***oxidative stress***") መጠን ለመለካት ነው::

የጥናቱ ሂደት፡- በዚህ ጥናት ለመሳተፍ ፍቃደኛ ከሆኑ ከእርሶ የምጠበቁ የሚከተሉት ናቸው::

1. ምርምሩን ለመስራት የሚያፈልገው የደም መጠን ፣ ደም ጥናቱን ለመስራት ብቻ እነሚውል::
2. የጥናቱ ባለቤት ትናቱን በተመለከተ አንዳንድ ጥያቄዎችን ሊጠይቅ ይችላል::

ጉዳት፡- ከዚህ ጥናት ጋር በተያያዘ በጤናም ሆነ በሚያገኙት ተገቢ ህክምና ምንም አይነት ጉዳት ስለማያስከትል አይስጉ::

ሚስጥራዊነት፡- ማንኛውም ከዚህ ጥናት ጋር የተያያዘ የግል መረጃ ሚስጥራዊነት የተጠበቀ ነው:: ስለዚህ የጥናቱ መረጃ ይፋ የሚሆነው ለረሶ ብቻ ነው:: ስለሚወሰደው ማንኛውም መረጃዎች ሆነ የጥናት ውጤት ለማሰራጨት በስም ሳይሆን በሚስጥር (ኮድ) የሚመዘገብ ይሆናል::

የተሳትፎ መብት፡- በዚህ ጥናት መሳተፍ ሙሉ በሙሉ በርሶ ፍቃድ የተመሰረተ መሆኑን ልናሳስን እንወዳለን:: በመሆኑም በማንኛውም ግዜ ምንም ዓይነት ምክንያት ሳይሰጡ ከጥናቱ ራስን የማግለል መበት የተጠበቀ ነው:: የሰጡት ደም ለዚህ ጥናት እንደሚውል ማድረግ በእርሶ ሙሉ ፈቃድ ብቻ ሲሆን በጥናቱ ላይ ለመሳተፍ መወሰን ወይም አለመወሰን መድሐኒት ወይም ሌላ የጤና አገልግሎት የማግኘት መብት አሁንም ሆነ ለወደፊቱ ምንም አይነት ተፅእኖ አያሳድርብትም::

Annex V: Consent form

I volunteer to participate in a research project conducted by Yimenashu Mamo from Addis Ababa University. I understand that the project is designed to gather information about Investigation oxidative stress different stages of breast cancer patients in Tikur Anbesa hospital, I will be one of approximately 100 people being participated for this research. I understand that I will not be paid for my participation. I may withdraw and discontinue participation from the study at any time without penalty. I understand that the researcher will not identify me by name in any reports using information obtained from this interview and that my confidentiality as a participant in this study will remain secure. Subsequent uses of records and data will be subject to standard data use policies which protect the anonymity of individuals and institutions.

I have been given a copy of this consent form and I have read and understand the explanation provided to me. I have had all my questions answered to my satisfaction, and I voluntarily agree to participate in this study.

Signature of subject_____ Signature of investigator _____
(Participant)

Date_____

Witness

Annex VI: consent form Amharic version

የዚህ ጥናት መሰረታዊ ዓላማ እና ሌሎች መረጃን በሚገባ ተገንዝቢያለሁ፡፡ ጥናቱ በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል በሚገኙ በተለያዩ ደረጃ ላይ በሚገኙ የጡት ካንሰር ታማሚዎች ላይ በሚከሰተን oxidative stress በመለካት ከተለያዩ የጡት ካንሰር ደረጃዎች ጋር የተለያዩ አመላካቾችን በመጠቀም ማወዳደር እንደሆነ በሚገባ ተገንዝቢያለሁ፡፡ ተሳትፎዬ በፍቃደኝነት ላይ ብቻ የተመረኮዘ እንደሆነም ተረድቻለሁ፡፡ ማንኛዉም ሰብዓዊም ሆነ ህጋዊ መብቴ ሳይነካ ከጥናቱ ራሴን ማግለል እንደምችልም እንደሆነ፡፡ ስለ ጥናቱ ዝርዝር ጉዳይ በግልፅ ከተረዳሁት ባሻገር በተጨማሪ ማብራሪያ ብፈልግ መጠየቅ እንደምችልም አዉቄያለሁ፡፡

የጥናቱም ባለቤት የዚህ የጥናቱ መረጃ ይፋ የሚሆነው ለእኔ ብቻ እንደሆነ እና ስለሚወሰደው ማንኛውም መረጃዎች ሆነ የጥናት ውጤት ለማሰራጨት በስም ሳይሆን በሚስጥር (ኮድ) እንደሆነም ተረድቻለሁ፡፡ በመሆኑም በፈቃዴ የዚህ ጥናት አካል እንድሆን ስፈልግ የምጠብቅብኝን ሁሉ ለሚድረግ በመወሰን መሆኑን በፊርማዬ አረጋግጣለሁ፡፡

የምስጥር ቁጥር	ቀን	ፊርማ
የጥናቱ ባለቤት ስም	ቀን	ፊርማ

ምስክር

Annex VII: Preparation of reagents and stock solutions

Preparation of TAC assay reagents

Reagent 1(R1): 0.4 mol/l acetate buffer solution (pH 5.8) was prepared (final concentration: 0.4 mol/l). Glacial acetic acid (22.8 ml) was diluted to 1000 ml with distilled water (final concentration: 0.4 mol/l). Sodium acetate solution (940 ml) was mixed with 60 ml of the acetic acid solution; the pH of the acetic acid–sodium acetate buffer was 5.8. The buffer solution was kept at 4 ^{0}C.

Reagent 2 (R2): **A**cetate buffer solution **(**30 mmol/l, pH 3.6) was prepared (final concentration: 30 mmol/l). Glacial acetic acid (1.705 ml) was diluted to 1000 ml with distilled water (final concentration: 30 mmol/l). The sodium acetate solution (75 ml) was mixed with 925 ml of the acetic acid solution; the pH of the acetic acid–sodium acetate buffer was made to 3.6. Then, 278 µl of H_2O_2 solution (35%) was diluted to 1000 ml with the buffer solution (final concentration, 2 mmol/l). Then 0.549g ABTS was dissolved in 100 ml of prepared solution (final concentration: 10 mmol/l). After 1 hour of room temperature incubation, the characteristic color of $ABTSS^+$ appeared.

Trolox standards graph preparation: 10 mL of the 100 mM (100 nmole/mL) Trolox standard solution was diluted within 990 mL of distilled water to prepare a 1 mM (1 nmole/mL) standard solution. 0, 2, 4, 6, 8, and 10 mL of the 1 mM ascorbic acid standard solution were added into well plate, generating 0 (reagent blank), and 200, 400, 600, 800, and 1000 mmol/well standards. Distilled water was added to each well to bring the volume to 100 ml.

Table2: - Different absorbance of different concentration of Trolox

Standard	Absorbances	Conc.(mmol)
Standard -1	0.073	0
Standard -2	0.065	200
Standard -3	0.05	400
Standard -4	0.0436	600
Standard -5	0.0349	800
Standard -6	0.0175	1000

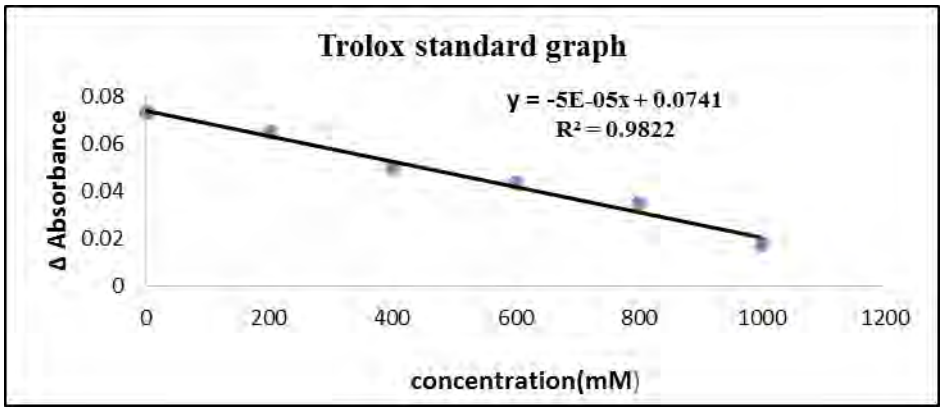

Figure 2:-Trolox standard graph

Preparation of stock solutions of reduced glutathione

Preparation of stock solutions: 0.2M phosphate buffer was prepared by taking 42.4 ml of 0.2M sodium hydroxide solution into a 250 ml volume metric flask, to this 50ml of 0.2M potassium dihydrogen phosphate solution was mixed and total volume was made 200ml with sufficient quantity of distil water. One milli Molar (1 mM) of Glutathione (GSH) solution was prepared by taking/dissolving 15.375 milligram of L. Glutathione (GSH) in 50ml of 0.1N hydrochloric acid solution. DTNB 1mM solution was prepared by dissolving 19.8 mg into 50 ml of phosphate buffer pH 7.6.

Standard graph preparation: After preparing 1mM stock solution of GSH (Glutathione), its different concentrations (table no.1) were prepared and from each of the GSH dilution 200µl was taken and added into 2300µl of 0.2 M phosphate buffer pH 7.6 then 500µl of 1mM 5,5-Dithiobis, 2-nitro benzoic acid (DTNB) was added, these five mixtures were well shaken and incubated for five minutes. After incubations period, absorbance of each mixture was recorded at fixed wavelength λ max: 412 nm. DTNB blank was prepared by adding 500µl DTNB (5, 5-dithiobis-2-nitrobenzoic acid) to 2500µl phosphate buffer pH 7.6. Absorbance of DTNB was also taken at same fixed wavelength λ_{max} of 412nm. By subtracting absorbance of blank DTBN from absorbance of each of the mixture, a real absorbance of each mixture was obtained as shown in table.

Table 1: Different absorbance of different concentration GSH dilutions

Standard	Absorbance	Concentration (µM)
Standard -1	0.135091	13.33
Standard -2	0.266182	26.66
Standard -3	0.373696	40.00
Standard -4	0.518461	53.33
Standard -5	0.65552	66.66

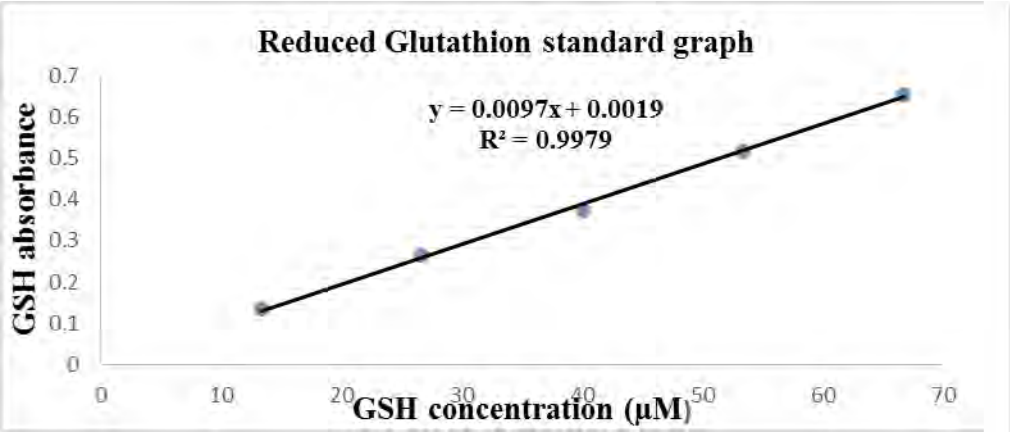

Figure 1:-Reduced Glutathione standard graph