ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICINE

DEPARTMENT OF MEDICAL BIOCHEMISTRY

BLOOD AND TISSUE ENZYME ACTIVITIES OF GDH, LDH, INDEX OF GLUTATHIONE AND OXIDATIVE STRESS AMONG BREAST CANCER PATIENTS ATTENDING REFERAL HOSPITALS OF ADDIS ABABA, ETHIOPIA: A COMPARATIVE CROSS SECTIONAL STUDY

MOHAMMED MEHDI ABRAR

A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOCHEMISTRY

MAY-2017

ADDIS ABABA, ETHIOPIA

BLOOD AND TISSUE ENZYME ACTIVITIES OF GDH, LDH, INDEX OF GLUTATHIONE AND OXIDATIVE STRESS AMONG BREAST CANCER PATIENTS ATTENDING REFERAL HOSPITALS OF ADDIS ABABA, ETHIOPIA: A COMPARATIVE CROSS SECTIONAL STUDY

Mohammed Mehdi Abrar

Advisors

1. Daniel Seifu, PhD
   Assistant professor of Medical Biochemistry
   Dean, School of Medicine
   College of Health Sciences
   Addis Ababa University, Addis Ababa, Ethiopia

2. Menakath Menon, PhD
   Department of Biochemistry, School of Medicine
   Addis Ababa University, Addis Ababa, Ethiopia.

3. Nebyou Seyoum, MD
   Department of Surgery, School of Medicine
   Addis Ababa University, Addis Ababa, Ethiopia.

4. Mahtime Bekele, MD
   Department of Surgery at St. Paul Millennium Medical College
   Addis Ababa, Ethiopia.

A thesis submitted to the school of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Medical Biochemistry

Addis Ababa University

School of Graduate Studies Department of Biochemistry

This is to certify that the thesis prepared by Mohammed Mehdi, entitled “Blood And Tissue Enzyme Activities of GDH, LDH, Index of Glutathione And Oxidative Stress Among Breast Cancer Patients Attending Referral Hospitals of Addis Ababa, Ethiopia: A Comparative Cross Sectional Study” is submitted in partial fulfillment of the requirements for the degree of Master of Science in Medical Biochemistry complies with the regulations of the University and meets the accepted standards with respect to the originality and quality.

Signed by the examining committee:

External examiner

________________________________ Signature _______________Date______________

Advisors

1. _____________________________ Signature _______________Date______________
2. _____________________________ Signature _______________Date______________
3. _____________________________ Signature _______________Date______________
4. _____________________________ Signature _______________Date______________

Distribution Agreement

This thesis is as a partial fulfillment of the requirements for the degree of master of science from Addis Ababa University, I hereby grant to Addis Ababa University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter known, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Mohammed Mehdi: _________________ Date: _________________________

Table of Contents

Table of Contents VI
List of Table IX
List of Figure X
Acknowledgments XI
Abbreviation/Acronym XII
Abstract XIV
1. INTRODUCTION 1
1.1. Background 1
1.2. Types of Cancer 1
1.3. Breast cancer 2
1.4. Types of Breast Cancer 2
1.5. Staging of Breast Cancer 4
1.6. Etiology of Breast Cancer 5
1.7. Metabolism in cancer cells 6
1.7.1. Lactate and Lactate Dehydrogenase (LDH) 7
1.7.2. Glutamate (Glu) and Glutamate Dehydrogenase (GDH) 8
1.8. Glutathione in Cancer Cells 9
1.9. Oxidative stress in Cancer cells 11
1.10. Statement of the problem 12
1.11. Significance of study 13
1.12. Hypothesis 13
2. OBJECTIVE 14
2.1. General Objective 14
2.2. Specific Objectives 14
3. METHODS AND MATERIALS 15
3.1. Study Area 15
3.2. Study Design 15
3.3. Study Period 15
3.4. Population 15
3.4.1. Source population 15
3.5. Sampling Method 16

3.6. Study variables 16
3.6.1. Dependent variables 16
3.6.2. Independent variables 16
3.7. Blood sample and Data Collection 16
3.7.1. Data collection procedure 16
3.8. Chemicals and Equipments used 18
3.9. Glutamate Dehydrogenase (GDH) Assay principle 18
3.10. Lactate Dehydrogenase (LDH) Assay principle 19
3.11. Total Glutathione (GSH) assay principle 19
3.12. Oxidized Glutathione (GSSH) assay principle 20
3.13. Total Oxidant Status (TOS) Assay principle 20
3.14. Total Antioxidant Capacity (TAC) assay principle 20
3.15. Determination of oxidative stress index (OSI) 21
3.16. Quality Assurance 21
3.17. Data processing and software used in statistical analysis 21
3.18. Ethical consideration 21
4. RESULTS 22
4.1. Socio-demographic profile 22
4.2. Clinical and Histopathological Findings 24
4.3. Biochemical Analysis 26
4.3.1. Serum and tissue enzymatic activity of glutamate dehydrogenase (GDH) 26
4.3.2. Serum and tissue enzymatic activities of lactate dehydrogenase (LDH) 27
4.3.3. Serum and tissue levels of Glutathione 28
4.3.4. Serum and tissue levels of TOS and TAC 29
4.3.5. Oxidative stress index (OSI) 29
4.3.6. Serum and tissue parameters with stages of breast cancer patients 30
4.3.7. Potential risk factors for breast cancer patients 32
5. DISCUSSION 34
6. CONCLUSION 40
7. RECOMMENDATION 41
8. STRENGTH AND LIMITATION 42
9. REFERENCE 43

10. ANNEXES 51
10.1. Questionnaire 51
10.2. Standard Curve 54
10.3. Information sheet in English Version 55
10.4. Information sheet in Amharic version 57
10.5. Informed consent form in English version 59
10.6. Informed consent form in Amharic version 60

List of Table

Table 1: Types of breast cancer with their unique characteristic feature…………………………2

Table 2: Socio-demographic profile of Breast Cancer Patients (BCA) and Control groups…….23

Table 3: Clinical and pathological profiles of breast cancer patients……………………….…...24

Table 4: Mean, Mean difference of serum parameters on control and BCA patients…………...25

Table 5: Mean, Mean difference of normal and tumor tissues of BCA patients……………..….26

Table 6: A one way Anova (Post-hoc) analysis of serum and tissue parameters………………..30

Table 7: Odds and p-value of variables to assess their risk to breast cancer …………………....32

List of Figure

Figure 1: Regulation of cancer metabolism by HIF-1 and c-Myc ……………………………….8

Figure 2: Role of glutamate in cancer cell......................……………………………….….……9

Figure 3: Multiple tumor supporter pathways promote GSH synthesis and regeneration…........10

Figure 4: Cellular redox status by a balance between levels of ROS inducers and ROS scavengers…………………………………………………………………………………..11

Figure 5: Chart showing workflow of sample processing ………………………………………17

Figure 6: Glutathione index in serum and tissue samples of breast cancer and control groups...27

Figure 7: Stress index in serum and tissue samples of breast cancer control group ………….... 28

Figure 8: Mean plots of OSI in serum and tissue samples of BCA patients ………….……….......31

Acknowledgments

My greatest and foremost indebtedness goes to the Almighty Allah who gives me the strength throughout my life.

Then, my first profound gratitude goes to my advisors Dr. Daniel Seifu, Dr. Menakath Menon, Dr. Nebyou Seyoum and Dr. Mahtime Bekele for their constructive comments, parental guidance and support during the onset to the completion of the study.

Secondly, the deepest gratitude also goes to Department of Biochemistry, Addis Ababa University, particularly to Dr. Daniel Seifu for his professional approach and support on administrative issues and Dr. Menon for his support on reagents.

Thirdly, it is my pleasure to express my heartfelt thanks to nurses and doctors who helped me during data collection at Yekatit 12 Hospital, Minilik Hospital, St. Paul Specialized Hospital Zewditu Memorial Hospital and Tikur Anbessa Specialized Hospital (TASH): without their support it would have been difficult to find all these participants of the study.

The last but not the least, I owe my respectful gratitude to the study participants and my friends, for their generous help and practical assistance during the study.

Beyond this many individuals who contributed for this study via their knowledge, ideas and time, I would like to take this opportunity to thank them, for their generous help.

Abbreviation/Acronym

ATP: Adenosine Triphosphate

BCA: Breast cancer

BRCA1: Breast Cancer Susceptibility Gene 1

BRCA2: Breast Cancer Susceptibility Gene 2

CHEK-2: Checkpoint kinase 2

CI: Confidence Interval

DCIS: Ductal Carcinoma *In situ*

DTNB: 5, 5'-Dithio-Bis (2-Nitrobenzoic Acid)

GAC: Activated Glutaminase C

GDH: Glutamate Dehydrogenase

GGT: gamma glutamyl transferase

GGTP: gamma glutamyl transpeptidase

GLAST: L-Glutamate/L-Aspartate Transporter

GLS1: Glutaminase

GSH: Reduced Glutathione

GSSH: Oxidized Glutathione

HER-2: Human Epidermal Growth Factor Receptor 2

HIF-1α: hypoxia inducible factor 1α

HR: Hormone Receptor

LCIS: Lobular Carcinoma *In situ*

LDH: Lactate Dehydrogenase

MCT1: Mono-carboxylate Transporters 1

MCT2: Mono-carboxylate Transporters 2

MHT: Menopausal Hormone Therapy

NADPH: Adenine Dinucleotide Phosphate

OSI: Oxidative Stress Index

OR: Odds Ratio

PALB2: Partner and Localizer of BRCA2

PDG: phosphate dependent glutaminase

PIG: phosphate independent glutaminase

PK: Pyruvate Kinase

PKM: Pyruvate Kinase Muscle Isozyme

PPP: Pentose Phosphate Pathway

PTEN: Phosphatase and Tensin homolog

ROS: Reactive Oxygen Species

Slc1A5: Alanine Serine Cysteine Transporter 2

STK11: Serine Threonine Kinase 11

TAC: Total Antioxidant Capacity

TKL1: Transketolase-1

TNM: Tumor, Nodal, Metastasis

TOS: Total Oxidative Status

TP53: Tumor Protein 53

αKG: Alpha Ketoglutarate

Abstract

Background: The exact cause of breast cancer is still unknown, however, it is a multifactorial and devastating disease. It is commonly diagnosed and second leading cause of death among women globally. Scholars are searching for an early marker for diagnosis, prognosis and potential target for treatment by looking it through many directions. One of the direction is its metabolism, all types of cancer need high energy input and substrates via aberrant metabolism for their survival. Therefore, the present study focused on investigating and examining pathways of glucose and glutamate metabolism and oxidative stress in breast cancer patients via analysis of glutamate dehydrogenase, lactate dehydrogenase, reduced and oxidized glutathione and the oxidative stress index of breast cancer patients.

Methodology: Comparative cross-sectional study was conducted from July, 2015 to May, 2017. A total of 54 (27 breast cancer patients and 27 control groups) participants were recruited. All were age-sex matched and mean age of breast cancer patients were 44.93 (25–68), whereas mean age of controls were 45.2 (24-66).

Result: Catalytic activities of glutamate dehydrogenase, lactate dehydrogenase and oxidative stress index were significantly increased both in serum (4.2mU/ml, 78.6mU/ml and 3.3:1 respectively) and cancerous tissues (1.4mU/ml), 111.7mU/ml and 2.15:1 respectively) of breast cancer patients as compared to serum of control groups (3.15mU/ml, 30.4mU/ml and 2.05:1 respectively) and non-cancerous tissue of breast cancer patients (0.92mU/ml, 70.5mU/ml and 1.1:1 respectively) ($P \leq 0.05$). Correspondingly, ratios of reduced to oxidized glutathione were significantly decreased both in serum (20:1) and cancerous tissues (23.5:1) of breast cancer patients when compared to serum of control groups (104.5:1) and non-cancerous tissues of breast cancer patient (70.9:1) ($P \leq 0.05$).

Conclusion: Catalytic activities of glutamate dehydrogenase and lactate dehydrogenase, and ratios of total oxidative status to total antioxidant capacity were significantly increased among breast cancer patients when compared to serum of control groups and non-cancerous tissues of breast cancer patients. While, ratios of reduced to oxidized glutathione and total antioxidant capacity in serum and tumor tissues of breast cancer were significantly decreased as compared to serum of control groups and normal tissues of breast cancer patients.

Key Words: Glutamate Dehydrogenase, Lactate Dehydrogenase, Reduced Glutathione, Oxidized Glutathione, Total oxidative status and Total antioxidant capacity and breast cancer

1. INTRODUCTION

1.1. Background

Cancer is complex, multifactorial and devastating diseases and it is characterized by its uncontrolled growth and spread of atypical cells (Fard *et al.,* 2015, Genetic Home Reference, 2015). During normal development, intricate genetic control systems regulate the balance between cell replication and death in response to growth signals, growth-inhibiting signals, and death signals. The losses of cellular regulation that give rise to most or all cases of cancer are due to mutations in two broad classes of genes: proto-oncogenes and tumor suppressor genes (Lodish *et al.,* 1995).

Currently, there is a thought that many familial cancers arise not exclusively from genetic makeup, but from the interplay among common gene variations, lifestyle and environmental risk factors. Only a small proportion of cancers are strongly hereditary, in that an inherited genetic alteration confers a very high risk (Antoniou *et al.,* 2014).

1.2. Types of Cancer

Cancers can be grouped according to the type of progenitor cells. According to the cell types, cancers are grouped into five main categories (1) Carcinoma (begins in the skin or in tissues that line or cover internal organs), (2) Sarcoma (begins in the connective or supportive tissues such as bone, cartilage, fat, muscle, or blood vessels), (3) Leukemia (starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and go into the blood), (4) Lymphoma and myeloma (begin in the cells of the immune system) and (5) Brain and spinal cord cancers (these are cancers of central nervous system). Cancers can also be classified according to their origin and organization in the body, such as breast cancer that starts in the tissues of the breast (Cancer research.UK, 2016).

1.3. Breast cancer

Breast cancer (BCA) is cancer that originates in the breast tissues of the body. Most breast cancers begin in the ducts of the breast that carry milk to the nipple (ductal cancers). Some start in the glands that make breast milk (lobular cancers). There are also other types of breast cancers (Sarcomas and lymphoma) that are less common (Girish *et al.,* 2014). Breast cancer is worst when it is malignant (cancerous) and that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body (Chaffer *et al.,* 2011).

1.4. Types of Breast Cancer

Based on the tissue types of origin of cancer cells, breast cancers can be divided into different types (some of them were listed in table 1). Most breast cancers are carcinomas, a type of cancer that starts in the cells (epithelial cells) that line organs and tissues like the breast. In fact, breast cancers are often a type of carcinoma grouped as adenocarcinoma, which is carcinoma that starts in glandular tissues. Other types of cancers can occur in the breast, too, such as sarcomas, which originates in the cells of muscle, fat, or connective tissues. In some cases, a single breast tumor can be a combination of different types of stem cells or be a mixture of invasive and *in situ* cancer (Girish *et al.,* 2014; Holowatyj *et al*., 2016; Schmadeka *et al.,* 2014).

Table 1: Types of breast cancer with their unique characteristic features

Types of Breast cancer	Unique features of the specific cancer type	References
1. *Ductal carcinoma Insitu (DCIS)*	• It refers to a condition where abnormal cells replace the normal epithelial cells of the breast ducts and may greatly expand the ducts • It is considered a noninvasive form of breast cancer	Liotta *et al.,* 2010; Girish *et al.,* 2014
2. *Lobular carcinoma Insitu (LCIS)*	• It refers to cells that seem cancer cells growing within the lobules of the breast • It is considered as marker for increased risk of developing invasive cancer	Logan *et al.,* 2015; Girish *et al.,* 2014
3. *Invasive or infiltrating Ductal Carcinoma (IDC)*	• It starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast • It may be able to spread (metastasize) to other parts of the body through the lymphatic system and bloodstream	Sharma *et al.,* 2010; Maier *et al.,* 2011; Girish *et al.,* 2014
4. *Invasive or infiltrating Lobular Carcinoma (ILC)*	• It starts in the milk-producing glands (lobules) • Like IDC, it can spread (metastasize) to other parts of the body	Sharma *et al.,* 2010; Maier *et al.,*

		2011; Girish *et al.,* 2014
5. *Luminal A (HR+/HER2-)*	• It express the estrogen receptor (ER+) and/or the progesterone receptor (PR+) but not HER2 (HER2-) • It tends to be slow growing and less aggressive than other subtypes	Holowatyj *et al*., 2016
6. *Triple Negative (HR-/HER2-)*	• It is an aggressive disease that affects a young population • It does not or negative overexpress human epidermal growth factor receptor 2 (HER2, ERBB2) and estrogen (ER) and progesterone receptor (PR) • It tends to grow and spread more quickly than most other types of breast cancer	Holowatyj *et al*., 2016; Schmadeka *et al.,* 2014; Girish *et al.,* 2014
7. *Luminal B (HR+/HER2+)*	• It is ER+ and/or PR+ and are further defined by being highly positive for Ki67 (an indicator of a large proportion of actively dividing cells) or HER2. • It tends to be higher grade and more aggressive than luminal A breast cancers	Holowatyj *et al*., 2016; Parise, and Caggiano, 2014
8. *HER2-enriched (HR-/HER2+)*	• It produce excess HER2 and do not express hormone receptors • It tends to grow and spread more aggressively than other breast cancers • It's are associated with poorer short-term prognosis compared to ER+ breast cancers	Holowatyj *et al*., 2016; Parise, and Caggiano, 2014
9. *Inflammatory Breast cancer (IBC)*	• It is uncommon and most virulent form of invasive breast cancer • Usually, there is no single lump or tumor. Instead, it makes the skin on the breast look red and feel warm • The affected breast may become larger or firmer, tender, or itchy • It has a higher chance of spreading and a bad prognosis than typical invasive ductal or lobular cancer	Dushkin *et al.,* 2011; Girish *et al.,* 2014
10. *Paget disease of the nipple*	• It starts in the breast ducts and spreads to the skin of the nipple and then to the areola, the dark circle around the nipple • The skin of the nipple and areola often appears crusted, scaly, and red, with areas of bleeding or oozing and it is rare	Sandoval-Leon *et al.,* 2013; Girish *et al.,* 2014
11. *Phyllodes tumor*	• It develops in the stroma (connective tissue) of the breast • It is a very rare type of breast tumor	Tan *et al.,* 2005; Girish *et al.,* 2014
12. *Angiosarcoma*	• It starts in cells that line blood vessels or lymph vessels • It occurs rarely in the breasts • It can also occur in the arms of women as a result of lymph node surgery or radiation therapy to treat breast cancer	Torres *et al.,* 2013; Girish *et al.,* 2014

1.5. Staging of Breast Cancer

Staging of breast cancer is based on the size or extent of the primary tumor and whether it has spread to nearby lymph nodes or other areas of the body. Proper staging is essential in determining the type of therapy and assessing prognosis. There are different staging systems used to classify breast cancer. Among those methods, systematic summary of staging is used for descriptive and statistical analysis of tumor registry data and particularly useful to look for any changes over time. According to this system, if cancer cells are present only in the layer of cells where they have been developed and have not spread to other areas, the stage is *in situ*. If cancer cells have penetrated or spread beyond the original layer of tissue, cancer has become invasive and is categorized as local, regional, or distant based on the extent of spread (Ozsaran and Alanyali, 2013).

A majority of breast cancers begin in the cell lines of a duct and named ductal cancer (DCIS). Some begin in the cell lines of lobule and named lobular cancers (LCIS), and a small percentage start on other types of tissues. After initiation of breast cancer, it may be metastatic. Metastatic breast cancer cell, first, enters into lymphatic vessels and begin to grow in lymph nodes, then into the bloodstream and spread (metastasized) such as to liver and bone. The more lymph nodes with breast cancer cells, the more probable it is that the tumor might be found in different organs also. Because of this, finding cancer in one or more lymph nodes often affects the treatment plan. Still, not only women with cancer cells in their lymph nodes develop metastases, but also some women have no cancer cells in their lymph nodes and later develop metastases (American Cancer Society, 2015; Ethiopian Lady, 2015).

The other method is TNM staging method; it is based on growth and spread of breast cancer. It has three terminologies (one) extent of the primary tumor (T), (two) absence or presence of regional lymph node involvement (N), and (three) absence or presence of distant metastases (M). Once the T, N, and M categories are determined, a stage of 0, I, II, III, or IV is estimated. Stage-0 is being *in situ*; abnormal cells are present but have not spread to nearby tissue. Stage-I, II, and III's are localized, in these cases, there is cancer, the higher the stage, the larger the tumor and the more it has spread into nearby tissues. And stage-IV is being the most advanced, where cancer has spread to distant parts of the body (Chiang and Massagué, 2008; Haick *et al.,* 2011; American Cancer Society, 2015 and National Cancer Institute, 2015).

1.6. Etiology of Breast Cancer

The exact etiology of breast cancer is still unknown; numerous risk factors associated with increased risk of breast cancer were studied. Those risk factors can be modifiable or non-modifiable. Owing to the tremendous impact of modifiable risk factors, it has been estimated that 50% of breast cancer is preventable (Stein *et al.,* 2004). These are weight gain especially after the age of 18 and/or being overweight or obese (for postmenopausal breast cancer), long-term use of hormone (combined estrogen and progestin), physical inactivity, alcohol consumption, heavy smoking particularly before their first pregnancy (Vanitha *et al,* 2014, Gordon-Dseagu, 2006; Kent, 2012, Bethea *et al.,* 2015).

On the other hand, there are non-modifiable risk factors for breast cancer. These are more of associated with of genetics of breast cancer patients. These are a family history of breast cancer, particularly having one or more affected first-degree relatives, inherited mutations (genetic alterations in $BRCA_1$ and $BRCA_2$, TP53, PALB2, CHEK2, PTEN, CDH1, c-Myc, and STK11). More recently, studies have revealed genetic features called single nucleotide polymorphisms (SNPs) as an increase in breast cancer risk (Maas *et al.,* 2016; Vanitha *et al,* 2014, American cancer society, 2015; Balmana *et al.,* 2011). However, these mutations are very rare in the general population (much less than 1%). There is a belief that most familial breast cancers are due to the interaction between lifestyle factors and more common variations in the genetic code that confers a small increase in breast cancer risk. Individuals with a strong family history of breast and/or certain other cancers, such as ovarian cancer, should consider counseling to determine if genetic testing is appropriate. Compared to women in the general population, there is a 7% risk of developing breast cancer on the age of 70, 55%-65% risk on $BRCA_1$ mutation carriers, 45%-47% risk on $BRCA_2$ mutation carriers. Mutations on the PALB2 gene appear to confer risk similar to $BRCA_2$ mutations (Vanitha *et al.,* 2014, American cancer society, 2015; Balmana *et al.,* 2011).

Furthermore, high breast tissue density, high bone mineral density, type 2 diabetes, certain benign breast conditions (such as atypical hyperplasia), high-dose radiation to the chest, menstrual periods that start early and/or end later in life, never having children, and having one's first child after age 30 are also risk factors for breast cancer (Zmuda *et al.,* 2001; Balmana *et al.,* 2011; Butt *et al* 2012; Vanitha *et al,* 2014, American cancer society, 2015).

1.7. Metabolism in cancer cells

A Nobel Prize winner Otto Warburg and his co-workers were the first on studying the metabolism of cancer in the 1920s. According to their thought, cancer is a metabolic disease. When normal cells are deprived 35% of their oxygen supply, they will either die or turn into cancer cells. Cancer cells are not like a normal cell, lack the “intelligence” as a result of their division, and will be uncontrolled. Such uncontrolled oncogene-driven proliferation of cancer cells and the absence of an efficient vascular bed causes low oxygen tension (hypoxia), forced cancer cells to live in conditions of aerobic glycolysis. Under aerobic conditions, tumor tissues can metabolize approximately ten-fold more glucose to lactate in a given time than normal tissues. Additional to the Warburg effect, cancer cells exhibit increased synthesis of protein, nucleotides and fatty acids (Kroemer *et al.,* 2008; Koppenol *et al.,* 2011; Warburg and Nguyen, 2015).

Following aerobic glycolysis, cancer cells produce bi-carbonic and lactic acids, lactate being the primary end product of aerobic glycolysis. Such acidic condition, favor tumor invasion and suppress anticancer immune effectors. Lactate that is produced by tumor cells can be taken up by stromal cells, to regenerate pyruvate that either can be squeezed out to refuel the cancer cell or can be used for oxidative phosphorylation (Eales *et al;* 2016; Pouyssegur *et al.,* 2006; Koukourakis *et al.,* 2006; Swietach *et al.,* 2007; Fischer et al., 2007).

In addition, tumor cells can either metabolize glucose through the pentose phosphate pathway (PPP) to generate Nicotinamide adenine dinucleotide phosphate (NADPH) or use intermediates of the glycolytic pathway for anabolic reactions (glucose 6-phosphate for glycogen and ribose 5-phosphate synthesis, dihydroxyacetone phosphate for tri-acyl glyceride and phospholipid synthesis, and pyruvate for alanine and malate synthesis). NADPH ensures the cell’s antioxidant defenses against a hostile microenvironment, chemotherapeutic agents and for fatty acid synthesis. The non-oxidative part of the PPP is controlled by transketolase reactions, and the transketolase-1 isoform (TKL1) is overexpressed in multiple cancers. Most importantly, embryonic isoform of pyruvate kinase (PK), which dephosphorylates phosphoenol pyruvate (PEP) to pyruvate, is highly expressed in tumors yet is absent from adult tissues except adipocytes (Christofk *et al.,* 2008a; Mazurek *et al.,* 2005; Gatenby and Gillies, 2004; Foldi *et al.,* 2007; Langbein *et al.,* 2006).

1.7.1. Lactate and Lactate Dehydrogenase (LDH)

Lactate dehydrogenase is composed of two separately encoded subunits, A and B that combine to form binomial distributions of the five expected tetramers. LDH regulates glycolytic flux by converting pyruvate to lactate. Induced overexpression of the c-Myc gene is responsible for many of the changes that induce malignant changes. c-Myc induces increased glycolytic flux in cancer cells partly through transcriptional elevation of lactate dehydrogenase (LDH), a glycolytic enzyme required for c-Myc-driven growth (Fig 1). LDH is also overexpressed by the transcription factor, hypoxia inducible factor 1α (HIF-1) (**Fig. 1**), which is a central regulator of the metabolic response to hypoxia. HIF-1α is overexpressed in breast tumors and is positively associated with proliferation and therefore poor outcome in breast cancer. Finally, LDH activity is increased by estrogen and estrogen receptor-positive breast tumors express increased LDH levels relative to adjacent normal tissues (Pouysségur *et al.,* 2006; Marshall Thornburg et al., 2008; Miller *et al.,* 2012).

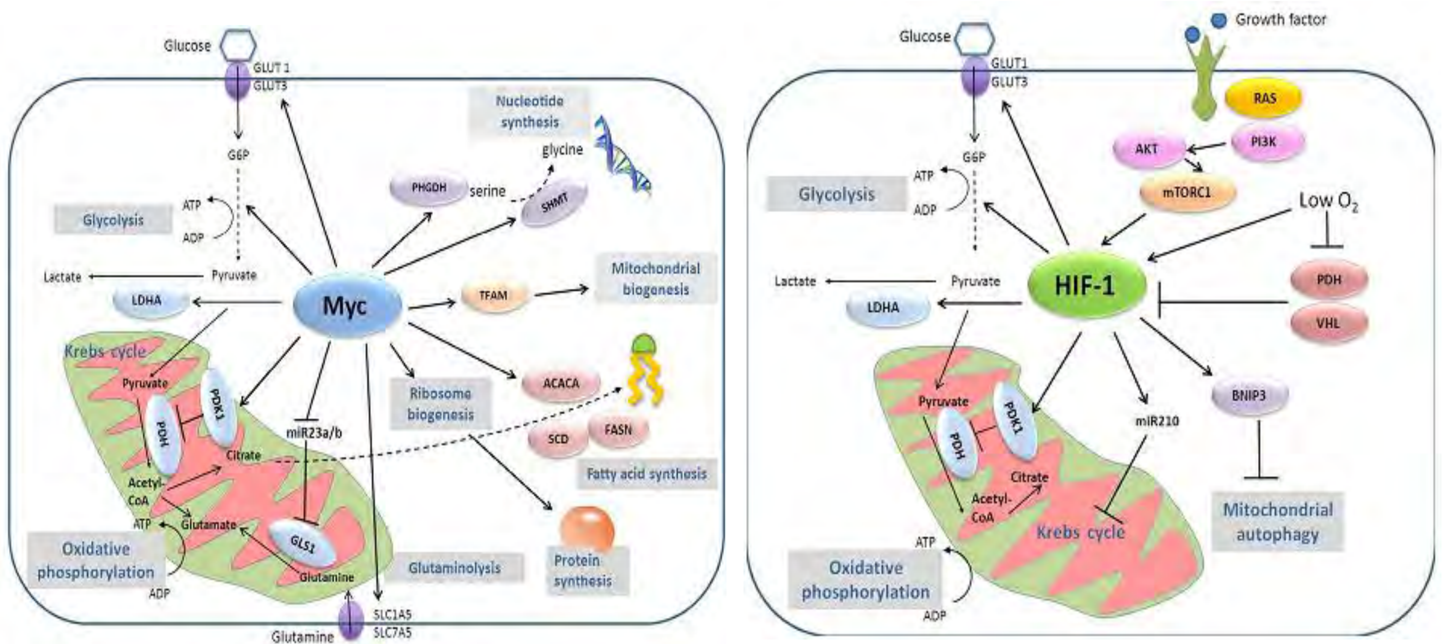


Figure 1: Regulation of cancer metabolism by HIF-1 and c-Myc (adopted from Iurlaro *et al.,* 2014). *Myc promotes cancer cell metabolism at several levels. Myc upregulates the glucose transporters GLUT1 and GLUT3 increasing glucose uptake. It induces several glycolytic enzymes such as the lactate dehydrogenase A (LDHA) resulting in lactate production. Like HIF-1, Myc induces pyruvate dehydrogenase kinase 1 (PDK1) expression, which prevents pyruvate entry into the mitochondria. Myc also regulates glutaminolysis: it upregulates glutamine transporters SLC1A5 and SLC7A5 and induces glutaminase 1*

(GLS1) expression. Myc also promotes biomass accumulation essential for proliferating tumor cells. It regulates ribosome biogenesis, mitochondrial biogenesis, and several enzymes involved in fatty acids synthesis such as acetyl-CoA carboxylase (ACACA), fatty acid synthetase (FASN), and stearoyl-CoA desaturase (SCD). Additionally, Myc regulates enzymes involved in nucleotide synthesis such as phosphoglycerate dehydrogenase (PHGDH) and serine hydroxymethyltransferase (SHMT) (Iurlaro et al., 2014).

1.7.2. Glutamate (Glu) and Glutamate Dehydrogenase (GDH)

Normally pyruvate (glucose) provides the Krebs cycle with pool intermediates while generated glutamate is transaminated to glutamine (NH_4^+/Gln ratio less than 1). Intracellular glutamate and alpha-ketoglutarate (αKG) are in near equilibrium and changes in Krebs cycle intermediates αKG as well as the redox state(NADPH/NADP), energy charge (ADP, ATP) and cell pH shift the glutamate dehydrogenase (GDH) enzyme catalyzed flux to net production or consumption of αKG (Friday *et al;* 2011; Koppenol *et al;* 2011).

In cancer cells, glucose is shunted into aerobic glycolysis cause a reduction in Krebs cycle intermediates "pulls" glutamate through GDH generating α-ketoglutarate (αKG). This is proved by the higher steady state of NH_4^+/Gln ratio greater than 1. Ammonium to alanine produced ratio will be increased. This will indicate the increased GDH and decreased ALT flux results in reduced intra-mitochondrial pyruvate (metabolized in the cytosol to lactate). Thus, the increased glutamate flux through GDH generates αKG while sparing ketoacid consumption (reduced transamination) (Friday *et al.,* 2012).

Glutamine is first taken up by the cell through a transporter (ASCT2), then Gln is either deaminated to Glu by cytoplasmic glutaminase-1 (GLS-1) or transported into the mitochondrial matrix by the Slc25a11 mitochondrial carrier and deaminated to Glu by glutaminase-2 (GLS-2). Consecutively, GDH converts Glu into αKG (**Fig 2**) (Villar *et al.,* 2015).

Regarding, αKG utilization in cancer cells, αKG is either carboxylated into isocitrate then to citrate and exported into the cytoplasm or αKG follows normal Krebs cycle until OAA is formed. The cytosolic enzyme ATP-Citrate-Lyase (ACL) splits citrate to oxaloacetate (OAA) and Acetyl-CoA. Acetyl-CoA is then utilized as a building block for lipid biosynthesis. Oxaloacetate is either converted into aspartate by aspartate transaminase (GOT2) and exported into the cytoplasm or

transformed into Asparagine and Arginine for protein synthesis (**Fig 2**) (Alberghina & Gaglio 2014; Wise *et al;* 2008; Bauer *et al;* 2012).

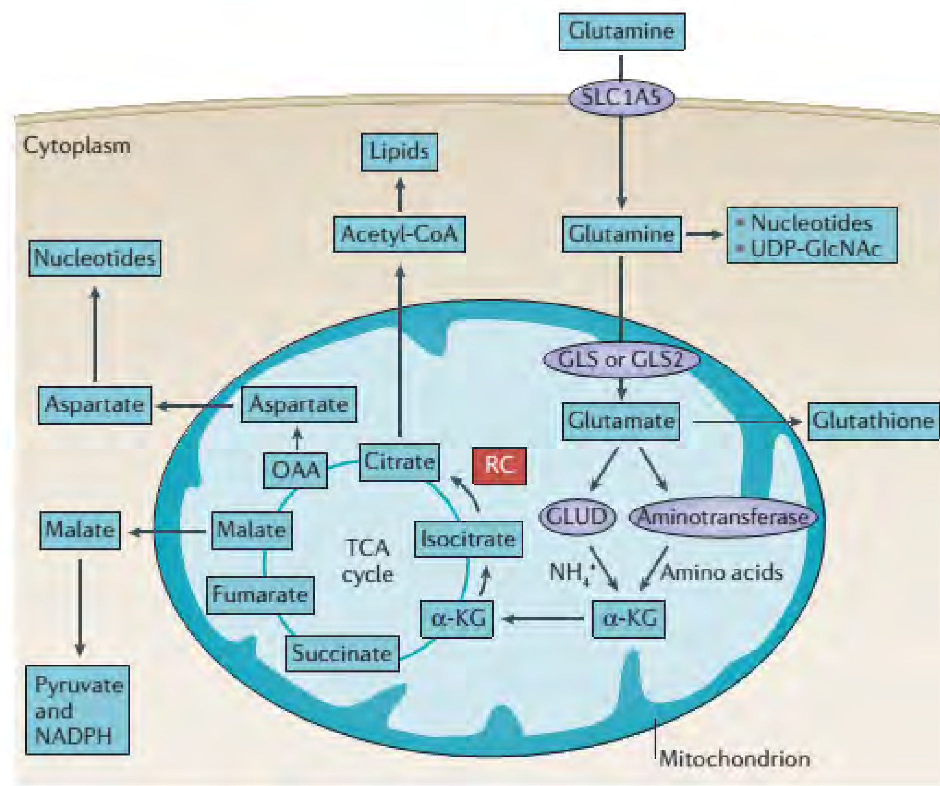


Figure 2: Role of glutamate in the cancer cell (adopted from Altman *et al.,* 2016). *Glutamate contributes to the synthesis of glutathione and has many other metabolic fates in the cell that have an impact on several inborn errors of metabolism. Reductive carboxylation (figure 2), seems to be a major source of carbon for lipid synthesis in cancer cells that are hypoxic, have constitutive hypoxia-inducible factor-α (HIFα) stabilization or have mitochondrial defects (Chen and Cui, 2015; Altman et al., 2016)*

1.8. Glutathione in Cancer Cells

Glutathione (GSH) is a tripeptide of glutamate, cysteine, and glycine. Its formation is highly dependent on glutamine substrate availability. It is the major thiol-containing endogenous antioxidant and serves as a redox buffer (approximately 5mM) against various sources of oxidative stress. In tumors, maintaining a supply of GSH is critical for cell survival because it allows cells to resist the oxidative stress associated with rapid metabolism, DNA-damaging agents, inflammation and other sources. In cancer cells, glutathione is particularly relevant in the

regulation of carcinogenic mechanisms; sensitivity against cytotoxic drugs, ionizing radiations, and some cytokines; DNA synthesis; and cell proliferation and death (Ortega *et al.,* 2011; Ballatori *et al.,* 2009; DeBerardinis & Cheng, 2010).

As shown in **figure 2 and 3**, glutamate contributes for proliferating tumor cells, they participate in bioenergetics, supports cell defenses against oxidative stress (NADPH and GSH) and glutamine complements glucose metabolism in the production of macromolecules. The concern in glutamine and glucose metabolism is heightened due to c-Myc and P53 controls uptake as well as degradation of glutamine and glucose respectively (DeBerardinis & Cheng, 2010; Rajagopalan & DeBerardinis, 2011).

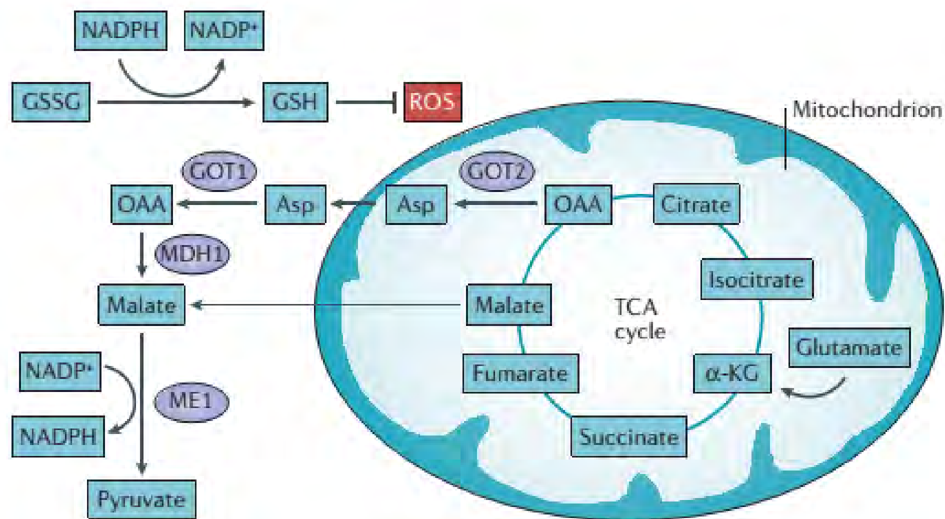


Figure 3: Multiple tumor supporter pathways promote GSH synthesis and regeneration (adopted from Altman et al., 2016). *In the first pathway, glutamine -derived malate is transported out of the mitochondria and is converted by malic enzyme 1 (ME1) into pyruvate, reducing one molecule of $NADP^+$ to NADPH. In the second pathway, found in mutant KRAS -transformed cells, aspartate that is produced from GOT_2 mediated transamination of glutamine derived oxaloacetate is transported out of the mitochondria. Aspartate is then converted in the cytosol back to OAA by GOT_1 and then to malate by malate dehydrogenase 1 (MDH1), which is in turn processed to pyruvate by ME1 to produce one molecule of NADPH (Gorrini et al., 2013; Altman et al., 2016) (figure 3).*

1.9. Oxidative stress in Cancer cells

Oxidative stress is defined as an imbalance or state of oxidation exceeds the antioxidant systems of the body (Yoshikawa and Naito, 2002). Reducing substances in the human body control the status of over oxidation, and a continuing imbalance in support of oxidation cause different problems when it beats the limit of such control. Free radicals and antioxidant can reinforce differing impacts on cells according to their concentration. Reactive oxygen species may participate in carcinogenesis through induction of gene mutations that result in cell damage and the consequences of signal transduction and transcription factors, and the redox status of cancer cells usually differs from that of normal cells (Yücel *et al.,* 2012). Because of metabolic and signaling aberrations, cancer cells exhibit elevated ROS levels and it is balanced by an increased antioxidant capacity (**Fig. 4**), which suggests that high ROS levels may constitute a barrier to tumorigenesis (Gorrini *et al.,* 2013).

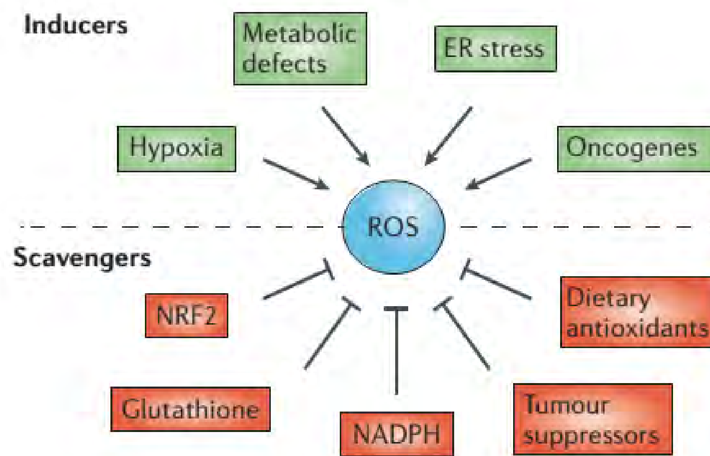


Figure 4: Determination of cellular redox status by a balance between levels of ROS inducers and ROS scavengers (Gorrini *et al.,* 2013). *The production of reactive oxygen species (ROS) can be induced by hypoxia, metabolic defects, endoplasmic reticulum (ER) stress and oncogenes. Conversely, ROS are eliminated by the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), the production of glutathione and NADPH, the activity of tumour suppressors (such as breast cancer susceptibility 1 (BRCA1), p53, phosphatase and tensin homolog (PTEN) and ataxia telangiectasia mutated (ATM)) and the action of dietary antioxidants (Gorrini et al., 2013).*

Despite, most cancer cells show lifted oxidative stress with expanded metabolic action and creation of ROS, many of chemotherapeutic medications induced apoptosis through oxidative stress. For instance, excellent antitumor medications cisplatin and adriamycin seem to create ROS at excessive levels, bringing about DNA damage and cell death. As malignancy cells are sensitive to oxidative stress, adjustment of SOD has been abused as a mechanism to specifically kill cancer cells (Klaunig *et al.,* 2010; Mahmood, 2010).

1.10. Statement of the problem

Globally among women, breast cancer is the most frequently diagnosed and second leading cause of cancer death. In the United States of America, from 2009 to 2013, the incident rate of breast cancer was 123.3/100,000 and death rate from 2010 to 2014 was 21.2/100,000. In 2017, it is estimated to diagnose new 252,710 invasive and 63,410 *Insitu* cases and 40, 610 death of breast cancer patients. Considering incidence trend of breast cancer from 2004 to 2013, invasive breast cancer seems to be stable in white women, but in black women, it has increased by 0.5% (American cancer Society, 2017).

Breast cancer is also the most commonly diagnosed and second leading cause of cancer death, surpassed only by lung cancer among African American women. From 2005 to 2009, the average annual breast cancer incidence rate in African American women was 118.1 cases per 100,000 women, 4% lower than in white women (123.2). Breast cancers diagnosed in African American women are more likely to have factors associated with poor prognosis, such as higher grade, advanced stage, and negative hormone (estrogen and progesterone) receptor status, than those diagnosed in white women (American Cancer Society, 2013).

Accurate cancer statistics are lacking in most countries in sub-Saharan Africa, but reports from some cancer registries within the region provide the incidence of breast cancer (Okobia, 2003). In Ethiopia, prevalence of breast cancer is increasing from time to time. From 1998 to 2010, it was 26% out of 9229 (Tigneh *et al.,* 2015), from 2011 to 2014 above 5701 (67 % female and 33% male) cancer cases were recorded and among those cases, breast cancer was the most commonly (33%) diagnosed diseases (Ethiopian Cancer Association (ECA), 2015; Kifle, 2014). Therefore, early diagnosis of the disease and identification of early metabolic markers for staging and therapy has become a necessity in Ethiopia.

1.11. Significance of study

Breast cancer is the most commonly diagnosed and the top leading cause of cancer death. Due to this, it is becoming a serious public health problem in Ethiopia. As a result, it is necessary to identify early markers for diagnosis, prognosis and a potential therapeutic target for breast cancer diseases. Therefore, this study is intended to identify early metabolic markers for diagnosis, prognosis and therapeutic target for breast cancer patients through evaluation of glutamate dehydrogenase and lactate dehydrogenase enzyme activities. Reduced and oxidized glutathione, as well as oxidative stress index of breast cancer tissue, could be compared with control groups and adjacent non-cancerous tissues of the same patients attending referral hospitals of Addis Ababa, Ethiopia.

1.12. Hypothesis

- ❖ Glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), reduced (GSH) and oxidized glutathione (GSSH), ratios of TOS to TAC (OSI) levels in serum and cancerous tissues of breast cancer patients is different from serum of control group and non-cancerous tissues of breast cancer patients.

2. OBJECTIVE

2.1. General Objective

- To evaluate glutamate and glucose metabolism through GDH and LDH enzyme activity, oxidant and anti-oxidative status among breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia.

2.2. Specific Objectives

- To assess glutamate dehydrogenase, lactate dehydrogenase, reduced glutathione, oxidized glutathione, total oxidative status and total antioxidant capacity within non-cancerous and cancerous tissues of breast cancer patients.
- To assess glutamate dehydrogenase, lactate dehydrogenase, reduced glutathione, oxidized glutathione, total oxidative status and total antioxidant capacity within serum samples of breast cancer patients and control groups.
- To explore mean difference of GDH and LDH enzyme activities, and concentration of GSH, GSSH, TOS and TAC within different stages of breast cancer patients.
- To assess potential risk factors for breast cancer diseases

3. METHODS AND MATERIALS

3.1. Study Area

The study was conducted in the five referral hospitals and one health center of Addis Ababa, Ethiopia. These were Tikur Anbessa Specialized Hospital (TASH), Zewditu Memorial Hospital (ZMH), St. Paul Specialized Hospital (SPH), Minilik the Second Hospital (MH), Yekatit 12 Hospital (YH) and Teklehaimanot health center (THC). Except for Teklehaimanot health center, all are referral hospitals of Addis Ababa, Ethiopia and have a high turnover of patients per day.

3.2. Study Design

A comparative Cross-sectional study was conducted to evaluate blood and tissue levels of glutamate dehydrogenase, lactate dehydrogenase, reduced and oxidized glutathione and the oxidative stress index among breast cancer patients.

3.3. Study Period

The study was conducted from July, 2015 to May, 2017

3.4. Population

3.4.1. Source population

The Source population was all cancer patients who visit the referral hospitals of Addis Ababa, Ethiopia during the study period.

3.4.2. Study Population

The study population was breast cancer patients who had a program of surgery at the referral hospitals of Addis Ababa, Ethiopia, during the study period.

3.4.3. Eligibility Criteria

3.4.3.1. Inclusion Criteria

Breast cancer patients who were volunteer and willingness to participate in the study were included

3.4.3.2. Exclusion Criteria

Breast cancer patients who were:

- Hypertensive
- Diabetic
- Liver diseased
- Recurrent Breast cancer patients were excluded from the study

3.5. Sampling Method

Purposive sampling technique was used to include all breast cancer patients during the study period. There was a well-structured questionnaire (annexure) which was used to select appropriate study participant, record clinical and socio-demographic data. Accordingly, 27 breast cancer patients and 27 control groups were participated.

3.6. Study variables

3.6.1. Dependent variables

- Glutamate Dehydrogenase (GDH)
- Lactate dehydrogenase (LDH)
- Reduced Glutathione (GSH)
- Total antioxidant capacity (TAC)
- Total oxidant status (TOS)
- Oxidized Glutathione (GSSH)
- Total Protein

3.6.2. Independent variables

- Age, Sex, Breast Cancer, BMI, Residence, Education level, Marital status, Child birth and Breast feeding

3.7. Blood sample and Data Collection

3.7.1. Data collection procedure

After a brief explanation, the patient's consent was asked for their willingness and participation. Then, before surgery, responses of questionnaire and blood samples were taken by professional nurses. After surgery, tumor and adjacent normal tissue samples were collected by attending surgeons of the surgery using Phosphate buffer saline (PBS).

3.7.1.1. Tissue Samples

A total of 54 tissue samples (27 tumor tissues and 27 normal tissues) were collected from 27 breast cancer patients. The collection of tumor and normal tissue was done by attending surgeons of the surgery at the operation room using phosphate buffer saline (PBS) and dimethyl sulfoxide (DMSO), and then stored at -80°c of the deep freezer until analysis. During the actual process, portions of the solid tumor, free of fat, connective tissue, necrotic debris, and blood, was cut into pieces of approximately 3 x 3 x 3 mm and frozen quickly (**Fig. 5**).

3.7.1.2. Blood samples

A total of 54 blood samples (27 breast cancer patients and 27 control groups) with a volume of 3-5ml were collected using serum separator tube (SST). The blood samples were centrifuged at 4000 rpm for five minutes then serum samples were harvested into Eppendorf tube. Then, it was stored in -80°c deep freezer until the analysis (**Fig. 5**).

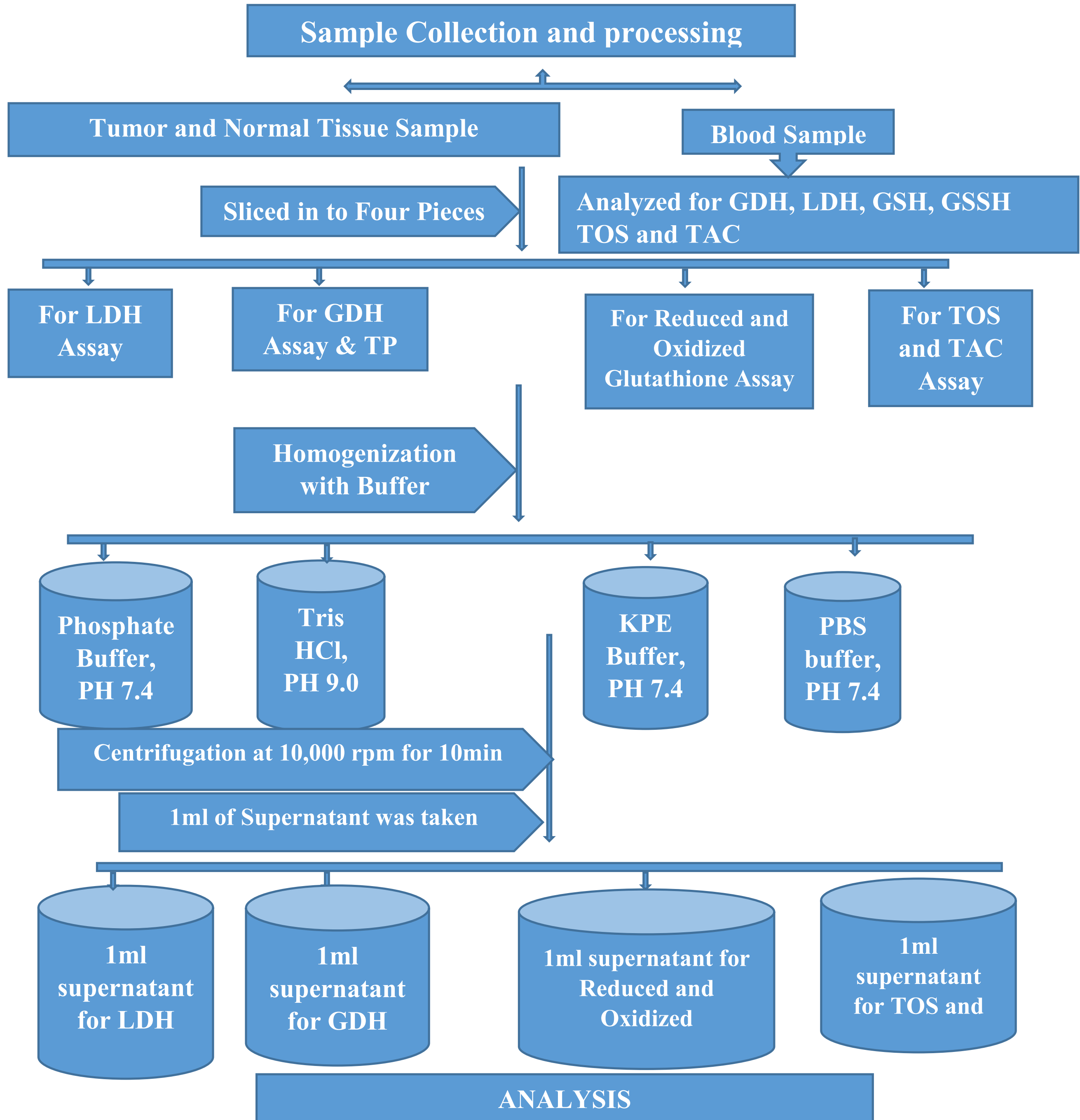


Figure 5: Chart showing workflow of sample processing

3.7.1.3. Homogenization of tissue sample

Tissue samples were thawed and sliced into five, approximately 50 to 100 mg wet weight. Each aliquot was homogenized in cold 0.05 M KPE buffer, pH 7.4; 0.1M phosphate buffer, pH 7.4; 0.1M Tris-HCl buffer, pH 9.0 and 0.1M phosphate buffer saline, pH 7.4 for the analyte of GSH and GSSH, LDH, GDH, TOS and TAC respectively. Then, the supernatant of the homogenates was taken for determinations of GSH and GSSH, LDH, GDH, TOS and TAC after centrifugation in 10,000 rpm for 10 min (fig. 5).

3.8. Chemicals and Equipments used

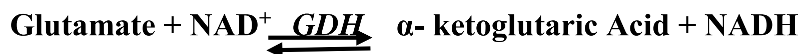
Glutamate dehydrogenase (GDH), Lactate dehydrogenase (LDH) Reduced form glutathione (GSH), Oxidized form glutathione (GSSH), Total oxidative status (TOS) and Total antioxidant capacity (TAC) were determined using the following kits and chemicals bought from Sigma-Aldrich, Merck and BDH Chemical Company. Potassium di-hydrogen orthophosphate (KH_2PO_4), Di-potassium hydrogen orthophosphate (K_2HPO_4), EDTA sodium salt, Sulfosalicyclic acid, Triton X-100, DTNB, β-NADPH, Glutathione reductase, reduced form Glutathione (GSH), Glutathione (disulfide form) (GSSG), Tri-ethanolamine and 2-Vinylpyridine, Xylenol orange, Sodium chloride (NaCl), Sulfuric Acid (H_2SO_4), Glycerol, Ferrous Ammonium Sulfate, o-dianisidine di-hydrochloride, sodium acetate (CH_3COONa), glacial acetic acid, 35%, H_2O_2 solution, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) and Trolox; (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

During laboratory analysis and processing of tissue and blood sample, different types of equipments were used. These were Eppendorf tube and falcon tube Centrifuge (Mulfifuge), ultraviolet/visible spectrophotometer (JENWAY, 6705, UK), ELISA plate reader (Biotest, 2001, Austria), Homogenizer (for tissues sample) (Heidolph, RZR 2100), Water path (GFL, 1002, Germany) and Magnetic stirrer.

3.9. Glutamate Dehydrogenase (GDH) Assay principle

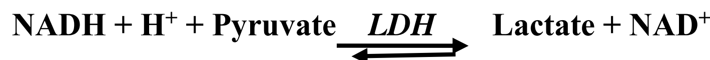
It was determined based on the principles of Botman. Glutamate dehydrogenase (GDH) catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate by producing NADH in forwarding reaction or consuming NADH in reverse reaction. Relative to the GDH activity present in the sample, the reduction of NAD^+ or oxidation of NADH is followed by spectrophotometrically at 340nm. One unit of GDH is the measure of an enzyme that will produce 1.0 µmole of NADH

every moment at pH 7.6 at 37 °C. The change of NAD+ to NADH is measured spectrophotometrically and is relative to the measure of Glutamate Dehydrogenase (Botman *et al.,* 2014). The test results were calculated based on the standard curve attached under annexure (annex 10.2) and expressed as mU/l.



3.10. Lactate Dehydrogenase (LDH) Assay principle

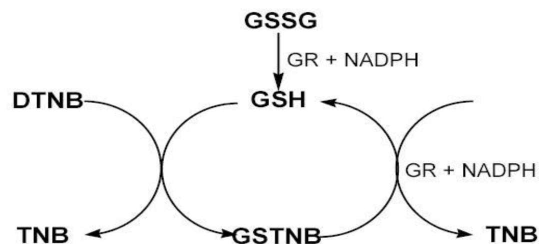
It was determined based on the principles of Vassault. It catalyzes the oxidation of L- lactate to pyruvate with the intervention of NAD as hydrogen acceptor reversibly. The absorbance of NAD+ measured with a spectrophotometer at 340 nm. According to beer-Lamberts law, in forwarding reaction, the consumption of NADH is directly proportional to the activity of LDH present in the sample (Vassault, 1983). The concentration of test results was calculated by using factor listed on LDH reagents kits.

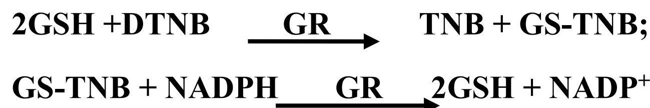


3.11. Total Glutathione (GSH) assay principle

It was determined based on the principles of Rahman. The assay depends on the reaction of GSH with DTNB that produces the TNB chromophore, and oxidized glutathione–TNB adduct (GS–TNB).

The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH in the sample. The disulfide product (GS–TNB) is then reduced by GR in the presence of NADPH, recycling GSH back into the reaction. Because GR reduces the GSSG formed into 2GSH, the amount of glutathione measured represents the sum of reduced and oxidized glutathione in the sample ([GSH] total = [GSH] + 2 × [GSSG]) (Rahman *et al.,* 2007). The test results were calculated based on the standard curve attached under annexure (annex 10.2).





3.12. Oxidized Glutathione (GSSH) assay principle

It was determined based on the principles of Rahman. The principle used GSSG reductase recycling method. By monitoring NADPH spectrophotometrically at a wavelength of 340 nm, the amount of GSSH was determined. The samples are treated with 2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine is neutralized with tri-ethanolamine. The test results were calculated based on the standard curve attached under annexure (annex 10.2).

3.13. Total Oxidant Status (TOS) Assay principle

It was determined based on the principles of Erel. In this process oxidants present in the sample oxidize the ferrous particle o-dianisidine complex to the ferric particle. The oxidation reaction is upgraded by glycerol molecules, which are richly present in the reaction medium. A colored compound is formed when the ferric ion reacts with xylenol orange in an acidic medium. The color strength, which can be measured spectrophotometrically at 560 nm wavelength, is correlated to the total quantity of oxidant molecules present in the plasma. The assay is aligned with hydrogen peroxide and the outcomes are expressed as far as µmolar hydrogen peroxide equivalent per liter (µmol H_2O_2 Eq/l) (Erel, 2005). The test results were determined based on the standard curve attached under annexure (annex 10.2).

3.14. Total Antioxidant Capacity (TAC) assay principle

It was determined based on the principles of Koracevic. In this technique, the hydroxyl radical, the most powerful natural radical, is generated by the Fenton reaction and it responds with the colorless substrate O-dianisidine to create the dianisyl radical, which is splendid yellowish-brown in color. Upon the addition sample, the oxidative responses started by the hydroxyl radicals present in the reaction are scavenged by the antioxidant agents present in the sample, keeping the color change and consequently giving a viable estimation of TAC (Koracevic *et al.,* 2001). The test results were calculated based on the standard curve attached under annexure (annex 10.2) and expressed as mmol Trolox Eq/l.

3.15. Determination of oxidative stress index (OSI)

It was calculated based on the method of Erel. The proportion of TOS to TAC is acknowledged as the oxidative stress index (OSI). For estimation, the subsequent unit of TAC is changed over to mmol/l, and the OSI value is computed (Erel, 2005).

- OSI (subjective unit) = TOS (μmol H_2O_2 Eq/l)/TAC (mmol Trolox Eq/l)

3.16. Quality Assurance

- There was a well-prepared data collection questionnaire to assess participant's demographic information.
- There was quality control for the clinical chemistry analyzers which was run daily in the morning before the actual sample running.
- There were a well-prepared working protocols for every parameter.

3.17. Data processing and software used in statistical analysis

All data's were checked, cleared and feed into Epi-data (version 3.5.1, 2008) and then exported to SPSS (version 22.0, 2012, America) software for statistical analysis. Descriptive analysis, Spearman correlation, and linear regression, independent sample T-test, one way ANOVA followed by post hoc analysis were used for this study. All data's were expressed in mean ± SD and $P \leq 0.05$ was considered as a statistically significant.

3.18. Ethical consideration

The ethical clearance was obtained from Biochemistry Department College of Health Sciences Addis Ababa University with protocol number 09/15 and meeting number DRERC 09/15. Furthermore, the consent of each participant was asked after a brief explanation of the objective of the study (see annexure).

4. RESULTS

4.1. Socio-demographic profile

A total of 54 (27 breast cancer patients and 27 control groups) participants were recruited. These were from five major referral hospitals of Addis Ababa, Ethiopia, and one health center. Those were Tikur Anbessa Specialized Hospital (TASH), St. Paul Specialized Hospital (SPH), Zewditu Memorial Hospital (ZMH), Yekatit 12 Hospital (YH), Minilik the second Hospital (MH) and Teklehaimanot Health Center (THC). Most of participants were from Tikur Anbessa Specialized Hospital 13 (48.1%) and St. Paul Specialized Hospital 6 (22.2%).

Socio-demographic profiles of participants are presented in table 2. All participants were female and their mean age was 44.93 with a minimum age of 25 to a maximum age of 68. Thirteen of them were less than or equal to 40 years old and fourteen of them were greater than 40 years old. Consecutively, sex and age-matched control samples were also collected.

Out of 27 of breast cancer patients, 17 (63.0%) were living in urban areas and 10 (37.0%) were living in rural areas. Twelve of breast cancer patients (44.5%) were illiterate, 17 (63.0%) of them married, 15 (55.6%) of them gave birth, had at least 1 and at most 4 children, 15 (55.6%) of them feed their children with breast milk, 14 (51.9%) of them used birth control, 16(59.3%) of them pre-menopausal and 3(11.1%) of them were obese (**Table 2**).

Table 2: Socio-demographic profile of Breast Cancer Patients (BCA) and Control groups at the five referral Hospitals and one Health Center of Addis Ababa, Ethiopia.

Socio-Demographic data of BCA and Control group		***BCA Patients (N=27)*** ***N (%)***	***Control Group (N=27)*** ***N (%)***
Age	≤40 Yrs.	13 (48.1)	14 (51.9)
	>40 Yrs.	14 (51.9)	13 (48.1)
Residence	Urban	17 (63.0)	19 (70.4)
	Rural	10 (37.0)	8 (29.6)
Education Level	Illiterate	12 (44.5)	0 (0.0)
	High school or less	11 (40.7)	14 (51.9)
	College and above	4 (14.8)	13 (48.1)
Marital status	Single	7 (25.9)	18 (66.7)
	Married	17 (63.0)	9 (33.3)
	Widowed	3 (11.1)	0 (0.0)
Child Birth	Yes	15 (55.6)	7 (25.9)
	No	12 (44.4)	20 (74.1)
No. of Children	0	12 (44.4)	20 (74.1)
	1-4	14 (51.9)	7 (25.9)
	≥5	1 (3.7)	0 (0.0)
Breast feeding	Yes	15 (55.6)	7 (25.9)
	No	12 (44.4)	20 (74.1)
Birth control	Yes	14 (51.9)	5 (18.5)
	No	13 (48.1)	22 (81.5)
Menopausal status	Pre	16 (59.3)	21 (77.8)
	Post	11 (40.7)	6 (22.2)
BMI	Under Weight (<18.5)	4 (14.8)	2 (7.4)
	Normal Weight (18.5-24.9)	14 (51.9)	18 (66.7)
	Over Weight (25-29.9)	6 (22.2)	4 (14.8)
	Obese (≥30)	3(11.1)	3(11.1)

4.1. Clinical and Histopathological Findings

Clinical and histopathological results of all breast cancer patients were studied, tabulated in table 3. From each breast cancer patients, tumor tissue, non-cancerous tissue (5cm away from cancerous tissue) and blood sample were collected. Histology of tumor tissues was graded as low grade or well differentiated 9 (33.3%), intermediate grade (intermediately differentiated) 10 (37.0%) and high-grade cases (poorly differentiated) 8 (29.6%). Staging of tumour tissues were done based on tumor size, all tumor tissues were classified into five stages 5 (18.5%) were stage zero, 4 (14.8%) were stage one, 7 (25.9%) were stage two, 8 (29.6%) were stage three, 3 (11.1%) were stage four. Based on invasiveness, tumor tissues were categorized into invasive ductal carcinoma 11 (40.7%), invasive lobular carcinoma 5 (18.5%), ductal carcinoma *in situ* 8 (29.6%), lobular carcinoma *in situ* 3 (11.1%) (**Table 3**).

Table 3: Clinical and pathological profiles of breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia.

Clinico-pathological data of BCA (N=27)		**N (%)**
Family History of Breast CA	Yes	7(25.9)
	No	20(74.1)
Location of Breast cancer (R/L)	Right Breast	17(63)
	Left Breast	10(37)
Tumor Size (pT)	pT1 (0.1-2 cm)	9(33.3)
	pT2 (2-5 cm)	8(29.6)
	pT3 (>5cm)	3(11.1)
	pT4 (extension to the chest wall/skin)	7(25.9)
Nodal Status (pN)	pN0	10(37.0)
	pN1	12(44.4)
	pN2	3(11.1)
	pN3	2(7.4)
Metastasis (M)	Mx	2(7.4)
	M0	23(85.2)
	M1	2(7.4)
Stage of breast CA	0	5(18.5)
	I	4(14.8)
	II	7(25.9)
	III	8(29.6)
	IV	3(11.1)
Grading	Low grade (Well-differentiated)	9(33.3)
	Intermediate grade (Moderately- differentiated)	10(37.1)
	High grade (Poorly- Differentiated)	8(29.6)
Histology of cancer	Invasive Ductal Carcinoma	11(40.7)
	Invasive Lobular Carcinoma	5(18.5)
	Ductal carcinoma in situ	8(29.6)
	Lobular Carcinoma in situ	3(11.1)

4.2. Biochemical Analysis

4.2.1. Serum and tissue enzymatic activity of glutamate dehydrogenase (GDH)

The catalytic activities of GDH in serum samples of breast cancer patients and control groups were significantly different ($P<0.05$) (95% CI (0.8 – 1.3)). Glutamate dehydrogenase in serum samples of breast cancer was 4.20±0.72 mU/l whereas, in control group, it was 3.15±0.69 mU/l. Similarly, catalytic activities of GDH in cancerous and non-cancerous tissues of breast cancer patients were assessed and significantly different ($P<0.05$) (95% CI (0.12-0.82)). The cancerous tissues had enzymatic activities of GDH than non-cancerous tissues (0.92 ± 0. 73 and 1.4 ± 0.88 mU/l respectively) (**Table 4 & 5**).

Table 4: Serum enzymatic activities of GDH and LDH, concentration of glutathione and the oxidative stress index of control groups (N=27) and breast cancer patients (N=27).

Serum Parameters	Control group	BCA patients	Mean Diff.	P. value	95% CI
GDH (mU/L)	3.15±0.69	4.20±0.72	1.04	0.000**	(0.8 – 1.3)
LDH (mU/L)	30.4±32.6	78.6±113	48.2	0.036*	(3.4 – 92.9)
GSH (µM per µg of protein)	20.9 ±2.6	10.2±2.9	-10.7	0.001**	(4.3 – 6.0)
GSSH (µM per µg of protein	0.2 ± 0.1	0.51 ± 0.2	0.31	0.001**	(0.8 – 1.2)
TOS (µmol H_2O_2 Equiv/l)	2.32±1.0	2.75±1.1	0.43	0.001**	(0.39 – 1.27)
TAC (mmol Trolox Equiv/l)	100.9±29.8	83.5±30.3	-13.65	0.017*	(-24.7 – -2.6)
OSI (Ratio of TOS/TAC*100)	2.3±1.5	3.3±1.7	1.0	0.006*	(0.32 – 1.67)

*The mean difference is significant at P. value ≤ 0.05

**The mean difference is significant at p. value ≤0.01

4.2.2. Serum and tissue enzymatic activities of lactate dehydrogenase (LDH)

Enzymatic activities of LDH in serum and tissue samples from breast cancer patients were investigated in comparison to serum samples of the control group and normal tissues of breast cancer patients. Serum LDH activities of breast cancer patients were significantly higher than serum samples of the control group (78.6±113 and 30.4±32.6 mU/L) ($P<0.05$) (95%CI (3.4 – 92.9) (table 4). Similarly, cancerous tissues had a higher LDH activities than non-cancerous tissues (111.7±23.2 and 70.5±10.7 mU/L) and it was statistically significant ($P<0.05$) (95%CI (-7.5 – 89.9) (**Table 4 & 5**).

Table 5: Tissue enzymatic activities of GDH and LDH, concentration of glutathione and the oxidative stress index of non-cancerous (N=27) and cancerous tissue (N=27).

Tissue Parameters	Normal Tissue	Tumor Tissue	Mean Diff.	P. value	95 % CI
GDH (mU/L)	0.92±0.73	1.40±0.88	0.5	0.011*	(0.12 – 0.82)
LDH (mU/L)	70.5±10.7	111.7±23.2	41.2	0.009	(-7.5 – 89.9)
GSH (µM per µg of protein)	14.9 ± 2.7	11.03 ± 2.0	-3.87	0.029*	(-0.2 – 0.8)
GSSH (µM per µg of protein	0.21 ± 0.1	0.47 ± 0.3	0.26	0.003**	(0.09 – 0.02)
TOS (µmol H_2O_2 Equiv/l)	2.1±0.9	3.5±1.1	1.4	0.001**	(0.33 – 1.22)
TAC (mmol Trolox Equiv/l)	188.9±26.7	161.6±50.8	-27.32	0.01**	(-47.42 – -7.2)
OSI (Ratio of TOS/TAC*100)	1.1±0.5	2.15±1.8	1.05	0.002*	(0.3 – 1.22)

*The mean difference is significant at P. value ≤ 0.05

**The mean difference is significant at p. value ≤0.01

4.2.3. Serum and tissue levels of Glutathione

The concentration of reduced and oxidized glutathione in serum and tissue samples of breast cancer patients were examined (**table 4 and 5**). Oxidized glutathione in serum of breast cancer patients were significantly ($P \leq 0.05$) higher than control groups (0.51 ± 0.2 and 0.2 ± 0.1 µM/µg of total protein, respectively). Similarly, cancerous tissues of breast cancer patient showed a significantly ($P \leq 0.05$) higher oxidized glutathione than non-cancerous tissue of breast cancer patients (0.47 ± 0.3 and 0.21±0.1 µM/µg of total protein, respectively).

Consecutively, serum of breast cancer patients was significantly lower in reduced glutathione as compared to serum of control groups (10.2±2.9 and 20.9 ±2.6 µM/µg of total protein respectively) ($P<0.05$). Correspondingly, reduced glutathione in cancerous tissue and non-cancerous tissue of breast cancer patients were statistically different ($P \leq 0.05$) tumor tissues had a lower reduced glutathione than normal tissue (11.03 ± 2.0 and 14.9 ± 2.7 µM/µg of total protein respectively). Furthermore, ratios of reduced (GSH) to oxidized glutathione (GSSH) in serum and cancerous tissues of breast cancer patients (19.9:1 and 32.3:1 respectively) was decreased as compared to serum samples of control group and non-cancerous tissue of breast cancer patients (30.7:1 and 75:1 respectively) (**Fig. 6**).

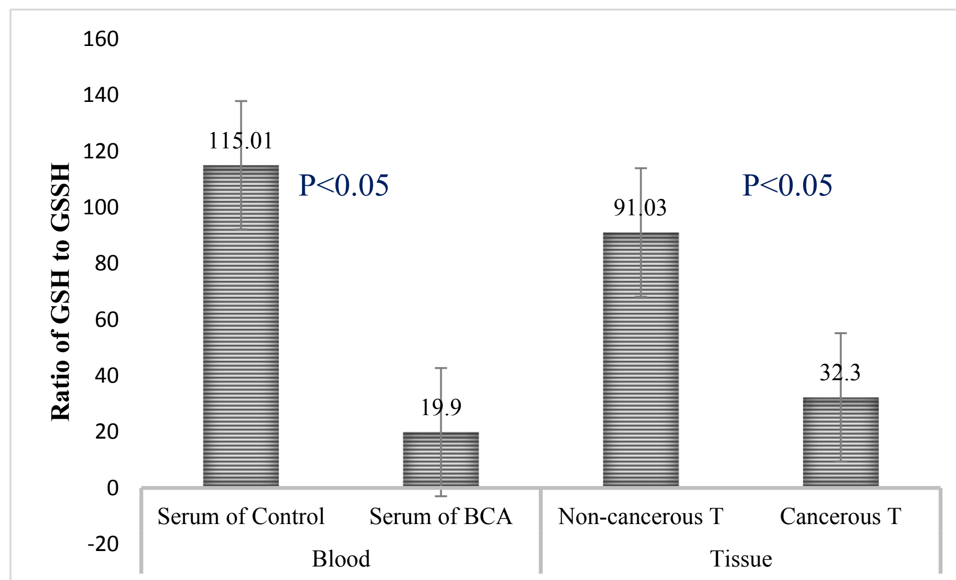


Figure 6: Glutathione index in serum and cancerous tissues of breast cancer (BCA) patients in comparison to serum of control group and non-cancerous tissue of breast cancer patients.

4.2.4. Serum and tissue levels of TOS and TAC

The concentration of total oxidative status (TOS) of breast cancer patients and control groups were examined (refer table 4 and5). Total oxidative status in serum of breast cancer patients (3.3±1.7 μmol H_2O_2 Equiv/l) were significantly ($P \leq 0.05$) higher than control groups (2.3±2.0 μmol H_2O_2 Equiv/l) (**Table 4 and 6**). Within tissue samples of breast cancer patients, total oxidative status in tumor tissues (2.15±1.8 μmol H_2O_2 Equiv/l) were significantly ($P \leq 0.05$) higher than normal tissue (1.1±0.5 μmol H_2O_2 Equiv/l) (**Table 4 & 5**).

In serum sample of breast cancer patients, there was a significantly ($P \leq 0.05$) lower amount of TAC concentration (83.5±30.3 mmol Trolox Equiv/l) than control groups (100.9±29.8 mmol Trolox Equiv/l). In tissue sample of breast cancer patients, TAC in tumor tissues were significantly ($P \leq 0.05$) lower in the concentration of TAC (161.6±50.8 mmol Trolox Equiv/l) than normal tissues (188.9±26.7 mmol Trolox Equiv/l) and their mean difference among tissue samples of breast cancer patient was statistically significant (**Table 4 & 5)**.

4.2.5. Oxidative stress index (OSI)

Likewise, OSI in serum and tissue samples of breast cancer patients and control groups were explored. Serum samples of breast cancer patients had a significantly higher OSI value (3.3±1.7) than control group (2.3±2.0) and the difference was statistically significant (P=0.006). Within tissues of breast cancer patients, cancerous tissues had a higher OSI (2.15±1.8) value than non-cancerous tissue (1.1±0.5) (P=0.002) (**Table 4, 5 & Fig. 7**).

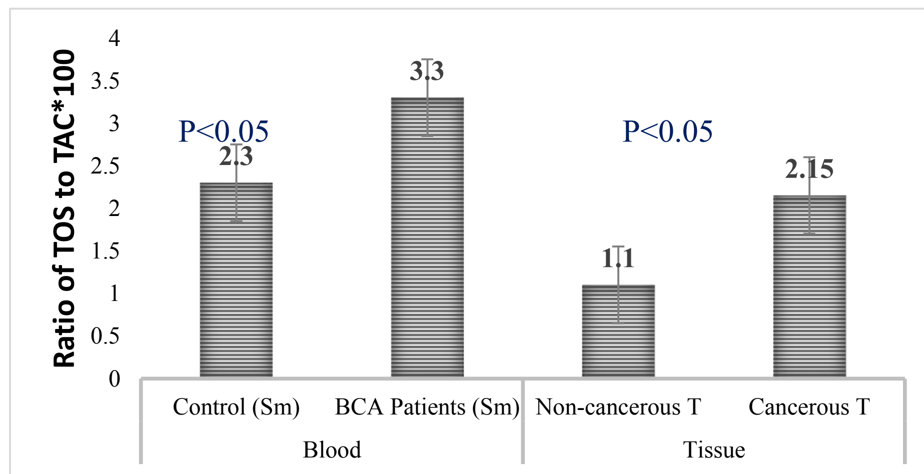


Figure 7: Oxidative stress index in serum and cancerous tissues of breast cancer (BCA) patients in comparison to serum of control group and non-cancerous tissue of breast cancer patients.

4.2.6. Serum and tissue parameters within stages of breast cancer patients

Blood and tissue parameters of GDH, LDH, GSH, GSSH, TOS, TAC and OSI were compared with stages of breast cancer patients (**refer table 6**).

Even though, it was not statistically significant (P>0.05), serum glutamate dehydrogenase enzyme activity was higher in stage zero (4.6±0.4 mU/L) and lower in stage four (3.7±0.45 mU/L). Whereas, tissue glutamate dehydrogenase enzyme activities was higher in stage three (1.9±1.1 mU/L) and lower in stage zero (0.7±0.2 mU/L) and it was statistically significant (P≤0.05).

The catalytic activities of serum lactate dehydrogenase were higher in stage four (341.8±41.4 mU/L) and lower in stage one (42.4±4.1 mU/L) and the mean difference of stage four with stage zero, one, two and three were statistically significant (P≤0.05) (table 6). The catalytic activities of tissue lactate dehydrogenase were higher in stage two (138.7±61.9 mU/l) and lower in stage zero (77.7±27.1 mU/L) however, their mean difference was not statistically significant (P>0.05).

Serum and tissue levels of total antioxidant capacity were lower in stage zero and four whereas in stage two and three there were a higher value but it was not statistically significant (P>0.05). Similarly, serum and tissue levels of total oxidative status were not consistent and fluctuate among the stages of breast cancer patients. The mean difference of total oxidative status in serum of stage zero of breast cancer patients was significantly different in comparison to the other stages I, III and IV (P=0.003, 0.002 and 0.045 respectively) (**Table 6**).

Table 6: A one way anova (Post-hoc) analysis of serum and tissue parameters in control subjects and pathologically confirmed breast cancer patients participated from five hospitals of Addis Ababa, Ethiopia (N=27).

Serum and tissue Parameters of BCA	Sample	Stages of Breast cancer Patients				
		Stage-0 (N=5)	Stage-I (N=4)	Stage-II (N=7)	Stage-III (N=8)	Stage-IV (N=3)
GDH	S	4.6±0.4	4.1±0.5	4.13±0.91	4.2±0.9	3.7±0.45
	T	0.7±0.2[a]	1.2±0.6	1.24±0.71	1.9±1.1[a]	1.78±1.15
LDH	S	77.7±27.1	80.9±38.7	138.7±61.9	57.6±5.4	90.0±11.7
	T	42.4±4.1[b]	63.8±5.1[b]	67.7±3.1[b]	131.3±8.4[b]	341.8±41.4[b]
GSH	S	5.4 ± 2.4	5.9 ± 0.5	5.8 ± 1.8	7.0 ± 3.3	6.3 ± 0.6
	T	1.0 ± 0.3	1.2 ± 0.5	2.0 ± 1.9	1.4 ±1.5	1.5 ± 0.7
GSSH	S	1.1 ± 0.5	1.2 ± 0.1	1.1 ± 0.4	1.4 ± 0.6	1.2 ± 0.1
	T	0.3 ± 0.1	0.3 ± 0.04	0.2 ±0.1	0.3 ± 0.1	0.2 ± 0.03
TOS	S	1.4±0.4[c]	3.4±1.3[c]	2.4±0.7	3.2±1.16[c]	2.8±0.6[c]
	T	3.2±0.3	2.3±0.4	3.3±1.6	2.5±1.2	2.4±0.8
TAC	S	0.90±0.29	0.84±0.14	0.87±0.21	0.90±0.34	0.77±0.47
	T	1.84±0.23	1.44±0.33	1.86±0.40	1.46±0.59	1.12±0.83
OSI	S	2.47±0.87	2.15±1.29	2.96±1.8	3.57±2.06	3.98±1.23
	T	2.1±0.87	1.13±0.84[d]	1.78±0.4	1.69±0.83	3.03±2.9[d]
Total Protein	S	212.1±14.4	209.7±8.6	197.3±8.6	210.9±11.6	215.3±4.34
	T	175.9±51.9	193.8±30.7	195.1±6.5	200.8±12.8	212.2±27.3

[a]mean difference of GDH between stage zero and three of tissue sample (p=0.029), [b]mean difference of LDH between stage four and zero, one, two and three of tissue sample (p=0.000, 0.001, 0.000 and 0.003 respectively), [c]between stage zero and one, three and four (P=0.003, 0.002 and 0.045 respectively) of blood sample and [d]mean difference of OSI among stage one and four in tissue of BCA (P=0.037) were statistically significant at $P < 0.05$. NB: Measuring units of GDH and LDH are in mU/L, GSH and GSSH were in µM/µg of total protein, TOS is in (µmol H_2O_2 Equiv/l), TAC is in (mmol Trolox Equiv/l) and total protein is in (µg/ml)

The oxidative stress index of breast cancer patients was analyzed and correlated with stage of cancer (**Fig. 8**). There was significantly higher oxidative stress in stage four of breast cancer patients than the other stages ($P<0.05$), whereas, it was lower in stage one.

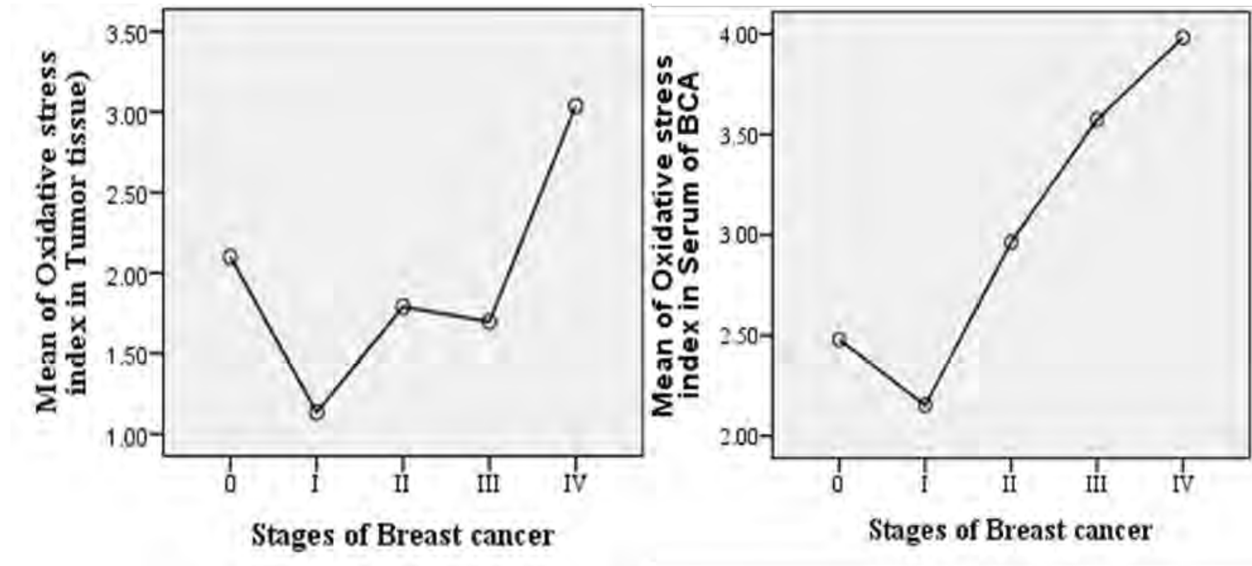


Figure 8: Mean plots of oxidative stress index in serum and tumor tissue samples of breast cancer patients

4.2.7. Potential risk factors for breast cancer patients

Different factors were observed to be associated with breast cancer risk (**Table 7**). Body mass index, marital status, child birth, breast feeding, birth control and menopausal status were assessed. Multinomial regression and crosstab analysis showed marital status [OR=0.186, 95% CI (1.826-16.823), P=0.003] and child birth [OR=0.571, 95% CI (1.134-11.253), P=0.027] had a lower risk for breast cancer than unmarried and never give birth women. Whereas, the use of birth control [OR=4.738, 95% CI (1.385-16.211), P=0.010] increased the risk of breast cancer significantly by 4.738 than non-birth control user women. The risk correlation of age with breast cancer patients was not statistically significant ($P>0.05$) since participants were age-sex matched.

Table 7: Odds ratios and p-value of independent variables to assess their risk to breast cancer

Variables*cases	N (%)		OR (95% CI)	P-value
	BCA patients	Control		
Age				
≤40	13 (48.2)	14 (51.8)	0.900 (0.004-1.302)	0.560
>40	14 (51.8)	13 (48.2)		
Body Mass Index (BMI)				
<18.5,	4 (14.8)	2 (7.4)		
18.5-24.9,	16 (59.3)	18 (66.7)	0.001 (0.517-1.935)	0.667
25-29.9,	6 (22.2)	4 (14.8)		
≥30)	3 (11.1)	3 (11.1)		
Residence				
Urban	17 (47.2)	19 (52.8)	0.716 (0.230-2.231)	0.773
Rural	10 (55.6)	8 (44.4)		
Education Level				
Illiterate,	12 (44.4)	0 (0)	0.291 (0.049-0.424)	**0.001***
Secondary or less,	11 (40.7)	13 (48.2)		
College	4 (14.8)	14 (51.8)		
Marital status				
Single	7 (25.9)	18 (66.7)		**0.003***
Married	17 (62.9)	9 (33.3)	0.186 (1.826-16.823)	
Widowed	3 (11.1)	0 (0)		
Child Birth				
Yes	15 (55.5)	7 (25.9)	0.571 (1.134-11.253)	**0.027***
No	12 (44.4)	20 (74.1)		
Birth control pills				
Yes	14 (51.8)	5 (18.5)	4.738 (1.385-16.211)	**0.010***
No	13 (48.2)	22 (81.5)		
Menopausal status				
Pre	16 (59.3)	21 (77.8)	0.416 (0.126-1.364)	0.143
Post	11 (40.7)	6 (22.2)		

* It is statistically significant at P-value <0.05

5. DISCUSSION

Currently, the prevalence of cancer is grown into a major public anxiety, as it is becoming the major cause of morbidity and mortality worldwide. More than 60 % of cancer cases occur in Africa, Asia, Central and South America. According to the International Agency for Research on Cancer (IARC), about 715,000 new cancer cases and 542,000 cancer deaths occurred in 2008 in Africa. In Ethiopia, there is no country-wide cancer registry, however based on Addis Ababa cancer registry; a total of 5701 cancer cases were registered from September 2011 to August 2014. Among those 3820 (67%) were females and 1881 (33%) were males. The most common type of cancers among females were cancers of the breast (33%), Cervix cancer (17%) and Ovary (6%), while among males cancers of colorectal (19%), Leukemia (18%) and prostate (11%) (Addis Ababa Cancer registry, 2014). Hence, an early biomarker for diagnosis, prognosis and a potential treatment target for breast cancer are required.

In the present study, serum and tissue levels of glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), reduced glutathione (GSH), oxidized glutathione (GSSH), total oxidative status (TOS) and total antioxidant capacity (TAC) were determined in search of a potential biomarker for diagnosis, prognosis and treatment target for breast cancer disease. Those serum and tissue parameters were studied on 54 (27 breast cancer patients and 27 age-sex matched apparently healthy control groups) participants.

As current study revealed that significantly higher enzymatic activities of GDH and LDH, ratios of TOS to TAC (OSI), and lower ratios of GSH to GSSH in serum and tissue samples from breast cancer patients were observed as compared to non-cancerous tissue of the same patients and serum samples of the control group.

Activities of GDH were significantly (P=0.011) increased (almost 1.5 times) both in serum and tumor tissue of breast cancer patients as compared to adjacent non-cancerous tissues of the same patient or serum samples of control groups. Furthermore, between stages of breast cancer, stage zero have the lowest and stage three have the highest activity of GDH in tumor tissues of breast cancer patients and the mean difference is statistically significant (P=0.029). These finding agreed with other studies done by Koukourakis *et al.,* (2008); Agrawal *et al.,* (2016); Liu *et al.,* (2015); Koppenol *et al.,* (2011), Lu *et al.,* (2010) & Toyokuni *et al.,* (1995). The possible reason for high

catalytic activities of GDH in cancer cells may be due to the fact that either it is important for redox homeostasis in cancer cells (Jin *et al.,* 2015) or over-expression of GDH promoted cell proliferation, migration, and invasion *in vitro*, whereas loss of function of GDH had the opposite effect (Liu *et al.,* 2015).

Previous works suggest that GDH enzymes are important in cancer cell either for synthesizing Krebs cycle intermediates (alpha-ketoglutarate and subsequent metabolite fumarate) or used for protein and fatty acid synthesis from citrate which originate from alpha-ketoglutarate. In addition to that, the substrate of GDH, glutamate itself is a substrate for antioxidant (GSH) and nucleotide synthesis in the cancer cell. These metabolic changes support the production of intermediates for cell growth and division and are regulated by both oncogenes and tumor suppressor genes, in a number of key cancer-producing pathways (Miller *et al.,* 2012; Altman *et al.,* 2016).

Similarly, the catalytic activities of LDH were significantly increased both in cancerous tissue and serum of breast cancer patients when compared to non-cancerous tissue of breast cancer patients and serum of control groups. The current study's result showed that mean values of LDH were significantly ($P<0.05$) lower in non-cancerous tissues (70.5±10.7) mU/L than tumor tissue (111.7±23.2 mU/L) of breast cancer patients. Patients with a higher clinical stage, had higher LDH activity than lower stages and there is a significant difference of mean between stage four (341.8±41.4) and stage zero (42.4±4.1 mU/L), stage I (63.8±5.1 mU/L), stage II (67.7±3.1 mU/L) and stage III (131.3±8.4 mU/L) ($P<0.05$). These finding agreed with the study of Talaiezadeh *et al.,* 2015 and Agrawal *et al.,* 2016.

The high activities of LDH in cancer cells may be due to the process of high cell proliferation, migration or invasion than normal cells. This is to say that large cancer cell population requires a higher and rapid energy source as compared to normal cell population. In order to meet this large and rapid energy demand, cancer cells use LDH activity as one option which is helpful for metabolic requirements and aerobic glycolysis of malignant cells. The possible mechanisms of high LDH activities in the cancer cell may be due to higher expressions of LDH gene in cancer cells as compared to normal cells (Talaiezadeh *et al.,* 2015; Agrawal *et al.,* 2016).

Generally, tissues have different rates of metabolic activity and oxygen consumption. When cells have a high production of reactive oxygen species than cellular antioxidant defenses attempts by the cells to remove these toxic species induces oxidative stress. Oxidative stress has long been

implicated in cancer development and progression (Limón-Pacheco *et al.,* 2009). The current study examined reduced glutathione (GSH), oxidized glutathione (GSSH), total oxidative status (TOS) and total antioxidant capacity (TAC) of serum and cancerous tissue of breast cancer patients in comparison to serum of control group and non-cancerous tissue breast cancer as a tool for assessing oxidative stress. The results showed that concentration of oxidized glutathione both in serum and tissue samples from breast cancer patients were significantly increased as compared to serum of control groups and non- cancerous tissues of breast cancer patients ($P<0.05$). Cancerous tissues (0.47 ± 0.3 µM/µg of total protein) have a higher mean value than non-cancerous tissue (0.21 ± 0.1 µM/µg of total protein). Whereas, the counterpart, concentration of reduced glutathione both in serum and cancerous tissue was significantly decreased when compared to serum of control groups and non- cancerous tissues of breast cancer patients ($P<0.05$), tumor tissues (11.03 ± 2.0 µM/µg of total protein) have lower mean value than normal tissue (14.9 ± 2.7 µM/µg of total protein).

In the same way, ratios of reduced (GSH) to oxidized glutathione (GSSH) in serum and cancerous tissues of breast cancer patients (19.9:1 and 32.3:1 respectively) was decreased as compared to serum samples of control group and non-cancerous tissue of breast cancer patients (30.7:1 and 75:1 respectively) (figure 4). These report agreed with the report of Perry *et al.,* (1993); Gamcsik *et al.,* (2013). Perry and his colleagues, (1993) reported that GSSH levels in primary breast tumors were more than twice the levels found in normal breast tissue, and levels in lymph node metastases were more than four times the levels found in normal breast tissue. Another group Gamcsik and his colleagues, (2013) reported that oxidized glutathione levels in breast tumors are higher than in disease-free breast tissue.

The possible justification for these results may be due to unusual levels of oxidative stress in breast cancer as oxidized glutathione in healthy tissue normally it is below 5% of the reduced from. This might be due to the content of GSH in some tumor cells is typically associated with higher levels of GSH-related enzymes, such as γ-glutamylcysteine ligase (GCL) and γ-glutamyl-transpeptidase (GGT) activities, as well as a higher expression of GSH-transporting export pumps (Perry *et al.,* 1993; Gamcsik *et al.,* 2013). Barranco and his colleagues, (2000) reported that the larger ratios of tumor glutathione to normal tissue glutathione, the poorer prognosis of cancer and less survival (Barranco *et al.,* 2000).

Moreover, the concentration of total oxidative status (TOS), total antioxidant capacity (TAC) and their ratios (OSI) in breast cancer patients and control groups were also investigated. The result revealed that TOS was significantly elevated both in serum and tumor tissues of breast cancer patients than serum of control groups and non-cancerous tissues of breast cancer patient (2.6±1.1 μmol H_2O_2 Equiv/l in the serum of breast cancer patients and 1.8±1.0 μmol H_2O_2 Equiv/l in serum control groups (P=0.001). Similarly, it was 2.8±1.1 μmol H_2O_2 Equiv/l in tumor tissues of breast cancer patients and 2.0±0.9 μmol H_2O_2 Equiv/l in normal tissue of the same breast cancer patients (P=0.001).

Correspondingly, breast cancer patients were a significantly lower concentration of total antioxidant capacity (TAC) both in serum (0.83±0.3 mmol Trolox Equiv/l) and in tumor tissue (1.61±0.5 mmol Trolox Equiv/l) than serum samples of control group (1.09±0.3 mmol Trolox Equiv/l) and in normal tissues (1.88±0.26 mmol Trolox Equiv/l) of breast cancer patients (P<0.05).

These finding agreed with the study of Sener *et al.,* (2007), Zowczak-Drabarczyk *et al.,* (2013), Júnior *et al.,* (2015); Panis *et al.,* (2015). Sener and his colleagues, (2007) found that TAC was (2.01±0.01 mmol Trolox Equiv/l) in patients with breast cancer and (2.07 ± 0.03 mmol Trolox Equiv/l) control groups (p<0.05) and Zowczak-Drabarczyk and his colleagues, (2013) found that TAC in the breast cancer patients was (1.35 mmol Trolox Equiv/l) in comparison to the controls (1.61 mmol Trolox Equiv/l) (P<0.05)). These findings are also supported by other works, which found a significantly higher value of oxidative status, as compared to control groups (Júnior *et al.,* 2015; Panis *et al.,* 2015). Furthermore, oxidative status in other types of cancer patients such as thyroid cancer and colorectal cancer patients reported with increased concentration of TOS (Sener et al., 2007; Zowczak-Drabarczyk *et al.,* 2013; Wang *et al.,* 2011; Wu *et al.,* 2017).

Consistently, ratios of TOS to TAC (OSI) in serum and tissue samples from breast cancer patients were significantly different as compared to serum samples of the control group and non-cancerous tissue of the same breast cancer patients. Serum samples of breast cancer patients have had a significantly higher ratio of total oxidative status to total antioxidant capacity (OSI) value (3.3±1.7) than the control group (2.3±1.5) (P=0.006). Likewise, tumor tissues of breast cancer patients had significantly higher value of OSI (2.15±1.8) than non-cancerous tissue (1.1±0.5) (P=0.002). This finding agreed with the study of Feng and his colleagues. They found a significantly higher values of OSI in breast cancer patients when compared to control groups (Feng *et al.,* 2012).

Surprisingly, even within the different stages of breast cancer patients, OSI value was different. Lower stages (0 and I) have lower values of OSI than the higher stages (II-IV) of breast cancer patients. Mean difference of OSI between stage one and four in tumor tissue of breast cancer patients is significant (P=0.037) different. This result is supported by the study of Zarrini and his colleagues, 2016. They reported that patients in advanced stage had lower serum antioxidant capacity and higher lipid peroxidation levels than control groups (Zarrini *et al.,* 2016).

The possible reason for high oxidative stress in breast cancer cell, it is may be due to oxygen radical production by the macrophages. In addition, tumor necrosis factor-α is secreted by tumour-associated macrophages and is known to induce cellular oxidative stress. Oxidative stress in cancer cells is useful either to increase mutation rate and accelerate tumor progression or to activate growth-promoting signaling pathways or to adapt oxidative stress, resulting in increased resistance to therapy or to increase blood supply to tumor cells or to increase the risk of invasion and metastasis (Brown and Bicknell, 2001, Jezierska-Drutel *et al.,* 2013).

Reczek and Chandel, (2016) explained the dual role of reactive oxygen species (ROS) in cancer. ROS can promote pro-tumorigenic signaling, facilitating cancer cell proliferation, survival, and adaptation to hypoxia. Conversely, ROS can promote anti-tumorigenic signaling and trigger oxidative stress induced cancer cell death (Reczek and Chandel, 2016).

Furthermore, oxygen radicals are powerful DNA damaging agents either ROS cause strand breaks or alterations in guanine and thymine bases or sister chromatid exchanges. This may inactivate additional tumor suppressor genes within tumor cells or further increase expression of proto-oncogenes. Genetic instability due to persistent carcinoma cell oxidative stress will, therefore, increase the malignant potential of the tumor (Brown and Bicknell, 2001).

Body mass index, marital status, child birth, birth control pills and menopausal status were also assessed through a standardized questionnaire. The current study shows that marital status, child birth have a lower risk for breast cancer and birth control have an increased risk for breast cancer. Results of giving birth [OR=3.571, 95% CI (1.134-11.253), P=0.027] agreed with the study of Nazir *et al.*, (2015). They stated that nulliparous (who never gave birth) women had significantly higher risk than parous (who gave birth) women (OR = 2.43, 95% CI = 1.22 – 4.84) however results of menopausal status disagreed with this study. In present study menopausal status and body mass index were not a significant risk factor for breast cancer. The current finding disagreed

with Nazir *et al.*, (2015), Masho *et al.,* (2015) and Pacholczak *et al.,* (2016). Nazir and his colleagues, (2015) reported that women with post-menopause are at higher risk (OR = 5.16, 95% CI = 2.59 – 10.29) than pre-menopause women (Nazir *et al.*, 2015). The possible reason for these differences may be due to different study design that is cross sectional or sample size of study participants in different laboratories.

Furthermore, results of birth control [OR=4.738, 95% CI (1.385-16.211), P=0.010] revealed that use of birth control pills has 4.738 times risk than non-user women. This is in agreement with the study of Fred Hutchinson Cancer Research Center. They stated that women taking certain formulations of birth control pills could face a 50 percent or higher increased risk of breast cancer than those not using oral contraceptives (Aleccia, 2014).

6. CONCLUSION

Enzymatic activities of glutamate dehydrogenase and lactate dehydrogenase, the concentration of reduced and oxidized glutathione and the ratio of total oxidative status and total antioxidant capacity (OSI) in serum and tumor tissues of breast cancer patients were determined. In conclusion, enzymatic activities of glutamate dehydrogenase and lactate dehydrogenase as well ratios of total oxidative status to total antioxidant capacity were significantly increased in serum and tumor tissues of breast cancer patients as compared to serum of control groups and non-cancerous tissues of breast cancer. However, ratios of reduced to oxidized glutathione were significantly decreased both in serum and cancerous tissues of breast patients as compared to serum of control groups and non-cancerous tissues of the same breast cancer patients. Furthermore, marital status, giving birth, breast feeding have a lower risk for breast cancer than unmarried women who never gave birth and non-breast feeding. Whereas birth control has a higher risk for breast cancer than non-user women.

Therefore, glutamate dehydrogenase, lactate dehydrogenase, and oxidative stress plays a critical role in breast cancer progression and may be an ideal therapeutic target for regulation of breast cancer disease.

7. RECOMMENDATION

- It is recommended to do large scale studies with larger sample size and proportion of different stages to confirm catalytic activities of glutamate dehydrogenase and lactate dehydrogenase, concentration of reduced and oxidized glutathione and oxidative stress among breast cancer patients
- Further studies of glutamate dehydrogenase and lactate dehydrogenase enzymes via immunohistochemistry (to detect genetic expression) and sequencing of their gene among breast cancer patients in comparison to control groups.
- Evaluating enzymatic activities of glutamate dehydrogenase and lactate dehydrogenase, concentrations of reduced and oxidized glutathione as well as oxidative stress with estrogen, progesterone and epidermal growth factor receptor among breast cancer patients and control groups.
- Assessing concentrations of reduced and oxidized glutathione as well as oxidative stress with enzymatic activities which are involved in glutathione synthesis in breast cancer patients and control groups and to recommend drug targeting at these sites.

8. STRENGTH AND LIMITATION OF THE STUDY

8.1.Strength of the Study

Measurement of enzyme markers in cancer tissues and correlations with stages is attempted for the first time in Ethiopia. It is hoped that assessment of serum and tumor tissue metabolism of glucose and glutamate, the concentration of reduced and oxidized glutathione, as well as oxidative stress of breast cancer patients, should favorably alter therapeutics indices of the patients. Therefore, this study is expected to provide the baseline information for further studies of breast cancer patient in Ethiopian hospitals, in relation to stages and grades.

8.2.Limitation of Study

The major limitation of this study was a small sample size of study participants and due to this the result of this study may not reflect total population of breast cancer patients in Ethiopia. The protocol of taking medicines of plant origin (traditional medicines) was not checked within the questionnaire.

9. REFERENCE

Addis Ababa City Cancer Registry, **2014**. Accessed on May 11, 2017 available at http://afcrn.org/membership/members/100-Addisababa.

Agrawal, A., Gandhe, M.B., Gupta, D. and Reddy, M.V.R., **2016**. Preliminary Study on Serum Lactate Dehydrogenase (LDH)-Prognostic Biomarker in Carcinoma Breast. Journal of clinical and diagnostic research: *JCDR*, 10(3), p.BC06.

Alberghina L. and Gaglio D. **2014**. Redox control of glutamine utilization in cancer. *Cell Death and Disease*. 5, e1561; doi:10.1038/cddis.2014.513.

Aleccia J.N., **2014**. Some birth control pills may boost breast cancer risk, Fred Hutch study finds. https://www.fredhutch.org/en/news/center-news/2014/08/Some-new-birth-control-raise-breast-cancer-risk.html. Accessed on january 16, 2015.

Al‑Muhtaseb, S.I., **2014**. Serum and saliva protein levels in females with breast cancer. Oncology letters, 8(6), pp.2752-2756.

Altman, B.J., Stine, Z.E. and Dang, C.V., **2016**. From Krebs to clinic: glutamine metabolism to cancer therapy. Nature Reviews Cancer, 16(10), pp.619-634.

American Cancer Society. **2013**. Cancer Facts & Figures for African Americans 2013-2014. Atlanta: American Cancer Society.

American Cancer Society. **2015**. Cancer Facts & Figures 2015. Atlanta: American Cancer Society.

Antoniou, A.C., Casadei, S., Heikkinen, T., Barrowdale, D., Pylkäs, K., Roberts, J., Lee, A., Subramanian, D., De Leeneer, K., Fostira, F. and Tomiak, E., **2014**. Breast-cancer risk in families with mutations in PALB2. New England Journal of Medicine, 371(6), pp.497-506.

Ballatori N., Krance S M., Notenboom S., Shi S., Tieu K. and Hammond C L. **2009**. Glutathione dysregulation and the etiology and progression of human diseases. *Biol Chem.,* 390, 191–214.

Balmana J., Dı´ez O., Rubio I. T., and Cardoso F. **2011**. BRCA in breast cancer: ESMO Clinical Practice Guidelines. *Ann Oncol.* 22(6): vi31–vi34.

Barranco, S.C., Perry, R.R., Durm, M.E., Quraishi, M., Werner, A.L., Gregorcyk, S.G. and Kolm, P., **2000**. Relationship between colorectal cancer glutathione levels and patient survival. *Diseases of the colon & rectum*, 43(8), pp.1133-1140.

Bauer DE., Jackson JG., Genda EN., Montoya MM., Yudkoff M., Robinson MB. **2012**. The glutamate transporter ,GLAST, participates in a macromolecular complex that supports glutamate metabolism. *Neurochem Int*. doi 10.1016/jneuint.2012.01.013.

Bethea, T.N., Rosenberg, L., Hong, C.C., Troester, M.A., Lunetta, K.L., Bandera, E.V., Schedin, P., Kolonel, L.N., Olshan, A.F., Ambrosone, C.B. and Palmer, J.R., **2015**. A case–control analysis of oral contraceptive use and breast cancer subtypes in the African American Breast Cancer Epidemiology and Risk Consortium. *Breast Cancer Research*, 17(1), p.1.

Brown, N.S. and Bicknell, R., **2001**. Hypoxia and oxidative stress in breast cancer Oxidative stress-its effects on the growth, metastatic potential and response to therapy of breast cancer. Breast cancer research, 3(5), p.323.

Butt, S., Harlid, S., Borgquist, S., Ivarsson, M., Landberg, G., Dillner, J., Carlson, J. and Manjer, J., **2012**. Genetic predisposition, parity, age at first childbirth and risk for breast cancer. *BMC research notes*, 5(1), p.1.

Cancer Research Uk: Types of cancer. Accessed on September 05, **2016**. Available at: http://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancer-starts/types-of-cancer

Chaffer, C.L. and Weinberg, R.A., **2011**. A perspective on cancer cell metastasis. *Science*, 331(6024), pp.1559-1564.

Chen, L. and Cui, H., **2015**. Targeting glutamine induces apoptosis: a cancer therapy approach. *International journal of molecular sciences*, 16(9), pp.22830-22855.

Chiang, A.C. and Massagué, J., **2008**. Molecular basis of metastasis. New England Journal of Medicine, 359(26), pp.2814-2823.

Christofk H R., Vander Heiden M G., Harris M H., Ramanathan A., Gerszten R E., Wei R., Fleming M D., Schreiber S L. And Cantley L C. **2008**. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumor growth. *Nature.* 452; 230–233.

Deberardinis R J. and Cheng T. **2010**. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* 29 313–324.

Dushkin, H. and Cristofanilli, M., **2011**. Inflammatory breast cancer. *Journal of the National Comprehensive Cancer Network*, 9(2), pp.233-241.

Eales, K.L., Hollinshead, K.E.R. and Tennant, D.A., **2016**. Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis*, 5(1), p.e190.

Erel, O., **2005**. A new automated colorimetric method for measuring total oxidant status. Clinical biochemistry, 38(12), pp.1103-1111.

Ethiopian Cancer Association (ECA). Breast cancer. Accessed on October 10, **2015**. Available at http://www.yeeca.org/Breast%20Cancer.htm.

Ethiopian Lady, How common is breast cancer? Accessed on October 10, **2015**. Available at http://www.ethiopianlady.com/cancer.html.

Fard, A.E., Zarepour, A., Zarrabi, A., Shanei, A. and Salehi, H., **2015**. Synergistic effect of the combination of triethylene-glycol modified Fe 3 O 4 nanoparticles and ultrasound wave on MCF-7 cells. *Journal of Magnetism and Magnetic Materials*, 394, pp.44-49.

Feng, J.F., Lu, L., Zeng, P., Yang, Y.H., Luo, J., Yang, Y.W. and Wang, D., **2012**. Serum total oxidant/antioxidant status and trace element levels in breast cancer patients. International journal of clinical oncology, 17(6), pp.575-583.

Fischer K., Hoffmann P., Voelkl S., Meidenbauer N., Ammer J., Edinger M., Gottfried E., Schwarz S., Rothe G., Hoves S., *et al.* **2007**. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood*. 109; 3812–3819.

Foldi M., Stickeler E., Bau L., Kretz O., Watermann D., Gitsch G., Kayser G., Zur Hausen A. And Coy J.F. **2007**. Transketolase protein TKTL1 overexpression: A potential biomarker and therapeutic target in breast cancer. *Oncol. Rep.* 17; 841–845.

Friday, E., Oliver, R., Welbourne, T. and Turturro, F., **2011**. Glutaminolysis and glycolysis regulation by troglitazone in breast cancer cells: Relationship to mitochondrial membrane potential. Journal of cellular physiology, 226(2), pp.511-519.

Friday, E., Turturro, F., Oliver III, R. and Welbourne, T., **2012**. Role of glutamate dehydrogenase in cancer growth and homeostasis. INTECH Open Access Publisher.

Gamcsik, M.P., Kasibhatla, M.S., Teeter, S.D. and Colvin, O.M., **2012**. Glutathione levels in human tumors. *Biomarkers*, 17(8), pp.671-691.

Gatenby R A. And Gillies R J. **2004**. Why do cancers have high aerobic glycolysis? *Nat. Rev. Cancer*, 4; 891–899.

Genetic home reference, 2015. Breast cancer. Accessed on October 14, **2015**. Available at http://ghr.nlm.nih.gov/glossary=malignancy.

Girish, C., Vijayalakshmi, P., Mentham, R., Rao, C.B. and Nama, S., **2014**. A Review On Breast Cancer. *Int J Pharm Bio Sc*, 4(2), pp.47-54.

Gordon-Dseagu, V., **2006**. Cancer and health inequalities: An introduction to current evidence. Cancer Research UK: London.

Gorrini, C., Harris, I.S. and Mak, T.W., **2013**. Modulation of oxidative stress as an anticancer strategy. *Nature reviews Drug discovery*, 12(12), pp.931-947.

Haick, H. and Shuster, G., Technion Research and Development Foundation Ltd., **2011**. Detection, staging and grading of benign and malignant tumors. *U.S. Patent Application* 13/697,554.

Holowatyj, A.N., Ruterbusch, J.J., Ratnam, M., Gorski, D.H. and Cote, M.L., **2016**. HER2 status and disparities in luminal breast cancers. Cancer medicine.

Iurlaro, R., León-Annicchiarico, C.L. and Muñoz-Pinedo, C., **2014**. Regulation of cancer metabolism by oncogenes and tumor suppressors. Methods Enzymol, 542, pp.59-80.

Jezierska-Drutel, A., Rosenzweig, S.A. and Neumann, C.A., **2013**. Role of oxidative stress and the microenvironment in breast cancer development and progression. Advances in cancer research, 119, p.107.

Jin, L., Li, D., Alesi, G.N., Fan, J., Kang, H.B., Lu, Z., Boggon, T.J., Jin, P., Yi, H., Wright, E.R. and Duong, D., **2015**. Glutamate dehydrogenase 1 signals through antioxidant glutathione peroxidase 1 to regulate redox homeostasis and tumor growth. *Cancer cell*, 27(2), pp.257-270.

Júnior, A.L.G., Paz, M.F.C.J., Silva, L.I.S.D., Sobral, A.L.P., Machado, K.D.C., Ferreira, P.M.P., Satyal, P., Freitas, R.M.D. and Cavalcante, A.A.D.C.M., **2015**. Serum oxidative stress markers and genotoxic profile induced by chemotherapy in patients with breast cancer: a pilot study. Oxidative medicine and cellular longevity, 2015.

Kent A. **2012**. Alcohol and Breast Cancer. *Rev Obstet Gynecol*. 2012; 5 (1):57.

Kifle M. 2014. Cancer on the rise in Ethiopia. accessed on October 10, **2015**. Available at http://www.ethiosports.com/2014/10/25/cancer-on-the-rise-in-ethiopia-dr-mahlet-kifle/

Klaunig J E., Kamendulis L M. and Hocevar B A. **2010**. Oxidative Stress and Oxidative Damage in Carcinogenesis. *Toxicologic Pathology,* 38**,** 96-109.

Koppenol W. H., Bounds P. L., Dang C. V. **2011**. Otto Warburg's contributions to current concepts of cancer metabolism. *Nature Review/cancer*, 11:325-337.

Koukourakis M I., Giatromanolaki A., Harris A.L. And Sivridis E. **2006**. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. *Cancer Res.* 66(2):632-7.

Koukourakis, M.I., Kontomanolis, E., Giatromanolaki, A., Sivridis, E. and Liberis, V., **2008**. Serum and tissue LDH levels in patients with breast/gynaecological cancer and benign diseases. *Gynecologic and obstetric investigation*, 67(3), pp.162-168.

Kroemer, G. and Pouyssegur, J., **2008**. Tumor cell metabolism: cancer's Achilles' heel. *Cancer cell*, 13(6), pp.472-482.

Langbein S., Zerilli M., Zur Hausen A., Staiger W., Rensch-Boschert K., Lukan N., Popa J., Ternullo M P., Steidler A., Weiss C., *et al.* **2006**. Expression of transketolase TKTL1 predicts colon and urothelial cancer patient survival: Warburg effect reinterpreted. *Br. J. Cancer.* 94; 78–585.

Limón-Pacheco, J. and Gonsebatt, M.E., **2009**. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 674(1), pp.137-147.

Liotta, L.A., Espina, V., Gallagher, R.I. and Edmiston, K., George Mason Intellectual Properties, Inc., **2010**. Malignant precursor cells from ductal carcinoma in situ lesions. *U.S. Patent Application* 13/258,119.

Liu, G., Zhu, J., Yu, M., Cai, C., Zhou, Y., Yu, M., Fu, Z., Gong, Y., Yang, B., Li, Y. and Zhou, Q., **2015**. Glutamate dehydrogenase is a novel prognostic marker and predicts metastases in colorectal cancer patients. *Journal of translational medicine*, 13(1), p.144.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P. and Darnell, J., **1995**. Molecular cell biology (Vol. 3). New York: Scientific American Books.

Logan, G.J., Dabbs, D.J., Lucas, P.C., Jankowitz, R.C., Brown, D.D., Clark, B.Z., Oesterreich, S. and McAuliffe, P.F., **2015**. Molecular drivers of lobular carcinoma in situ. *Breast Cancer Research*, 17(1), p.1.

Lu, W., Pelicano, H. and Huang, P., **2010**. Cancer metabolism: is glutamine sweeter than glucose? *Cancer cell*, 18(3), pp.199-200.

Maas, P., Barrdahl, M., Joshi, A.D., Auer, P.L., Gaudet, M.M., Milne, R.L., Schumacher, F.R., Anderson, W.F., Check, D., Chattopadhyay, S. and Baglietto, L., **2016**. Breast cancer risk from modifiable and nonmodifiable risk factors among white women in the United States. *JAMA oncology*, 2(10), pp.1295-1302.

Mahmood N A. **2010**. Oxidative Stress and Antioxidant Status in Colorectal Cancer and Healthy Subject. *Iraqi Journal of Cancer and Medical Genetics,* 3 (1)**,** 1-6.

Maier, J., Cohen, J. and Stewart, S., Chemimage Corporation, **2011**. Distinguishing between invasive ductal carcinoma and invasive lobular carcinoma using raman molecular imaging. *U.S. Patent*, 7; 956-996.

Marshall Thornburg J., KNelson K., Clem B F., Lane A N., Arumugam S., Simmons A., Eaton J W, Telang S. and Chesney J. **2008**. Targeting aspartate aminotransferase in breast cancer. *Breast Cancer Research*, 10 (5):R84 (doi: 10.1186/bcr2154).

Masho, S.W., Cha, S. and Morris, M.R., **2015**. Prepregnancy obesity and breastfeeding noninitiation in the United States: An examination of racial and ethnic differences. Breastfeeding Medicine, 10(5), pp.253-262.

Mazurek S., Boschek C B., Hugo F. And Eigenbrodt E. **2005**. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin. Cancer Biol.*15; 300–308.

Miller, D.M., Thomas, S.D., Islam, A., Muench, D. and Sedoris, K., **2012**. c-Myc and cancer metabolism. *AACR*, PP.5546-5553.

National Cancer Institute, staging of cancer, **2015**. Accessed on Febraruary 03/02/2017 available at https://www.cancer.gov.

Nazir, N., Waheed, A., Farhat, K., Ismail, M., Mansoor, Q. and Javed, G., **2015**. A case control study of risk factors associated with female breast cancer. Age, 187, pp.93-5.

Okobia M N. **2003**. Cancer Care in sub-Saharan Africa – Urgent Need for Population–based Cancer Registries. *Ethiop.J.Health Dev.* 17(2):89-98.

Ortega A L., Mena S. and Estrela J M. **2011**. Glutathione in Cancer Cell Death. *Cancers* 3, 1285-1310.

Ozsaran Z. and Alanyali S. D. **2013**. Staging of Breast Cancer. Principles and Practice of Modern Radiotherapy Techniques in Breast Cancer. *Haydaroglu A. and Ozyigit G. New York, Springer Science+Business Media*: 13-19.

Pacholczak, R., Klimek-Piotrowska, W. and Kuszmiersz, P., **2016**. Associations of anthropometric measures on breast cancer risk in pre-and postmenopausal women—a case-control study. Journal of physiological anthropology, 35(1), p.7.

Panis, C., Victorino, V.J., Herrera, A.C.S.A., Cecchini, A.L., Simão, A.N.C., Tomita, L.Y. and Cecchini, R., **2015**. Can breast tumors affect the oxidative status of the surrounding environment? A comparative analysis among cancerous breast, mammary adjacent tissue, and plasma. Oxidative medicine and cellular longevity, 2016.

Parise, C.A. and Caggiano, V., **2014**. Breast cancer survival defined by the ER/PR/HER2 subtypes and a surrogate classification according to tumor grade and immunohistochemical biomarkers. *Journal of cancer epidemiology*, 2014.

Perry, R.R., Mazetta, J., Levin, M. and Barranco, S.C., **1993**. Glutathione levels and variability in breast tumors and normal tissue. *Cancer*, 72(3), pp.783-787.

Pouysségur J., Dayan F. and Mazure N M. **2006**. Review article Hypoxia signalling in cancer and approaches to enforce tumor regression. *Nature.* 441; 437-443.

Rajagopalan K N. and Deberardinis R J. **2011**. Role of Glutamine in Cancer – Therapeutic and Imaging Implications. *J Nucl Med.,* 52, 1005–1008.

Reczek, C.R. and Chandel, N.S., **2016**. The Two Faces of Reactive Oxygen Species in Cancer. Annual Review of Cancer Biology, (0).

Sandoval-Leon, A.C., Drews-Elger, K., Gomez-Fernandez, C.R., Yepes, M.M. and Lippman, M.E., **2013**. Paget's disease of the nipple. Breast cancer research and treatment, 141(1), pp.1-12.

Schmadeka, R., Harmon, B.E. and Singh, M., **2014**. Triple-negative breast carcinoma. *American journal of clinical pathology*, 141(4), pp.462-477.

Sener, D.E., Gonenc, A., Akinci, M. and Torun, M., **2007**. Lipid peroxidation and total antioxidant status in patients with breast cancer. Cell Biochem Funct, 25(4), pp.377-82.

Sharma, G.N., Dave, R., Sanadya, J., Sharma, P. and Sharma, K.K., **2010**. Various types and management of breast cancer: An overview. *Journal of advanced pharmaceutical technology & research*, 1(2), p.109.

Stein, C.J. and Colditz, G.A., **2004**. Modifiable risk factors for cancer. *British Journal of Cancer*, 90(2), pp.299-303.

Swietach P., Vaughan-Jones R D., Harris A L. **2007**. Regulation of tumor pH and the role of carbonic anhydrase 9.*Cancer Metastasis Rev.* 26; 299–310.

Talaiezadeh, A., Shahriari, A., Tabandeh, M.R., Fathizadeh, P. and Mansouri, S., **2015**. Kinetic characterization of lactate dehydrogenase in normal and malignant human breast tissues. *Cancer cell international*, 15(1), p.19.

Tan, P.H., Jayabaskar, T., Chuah, K.L., Lee, H.Y., Tan, Y., Hilmy, M., Hung, H., Selvarajan, S. and Bay, B.H., **2005**. Phyllodes tumors of the breast. *American journal of clinical pathology*, 123(4), pp.529-540.

Tigeneh, W., Molla, A., Abreha, A. and Assefa, M., **2015**. Pattern of Cancer in Tikur Anbessa Specialized Hospital Oncology Center in Ethiopia from 1998 to 2010. Int J Cancer Res Mol Mech, 1, pp.1-5.

Torres, K.E., Ravi, V., Kin, K., Yi, M., Guadagnolo, B.A., May, C.D., Arun, B.K., Hunt, K.K., Lam, R., Lahat, G. and Hoffman, A., **2013**. Long-term outcomes in patients with radiation-associated angiosarcomas of the breast following surgery and radiotherapy for breast cancer. *Annuals of surgical oncology*, 20(4), pp.1267-1274.

Toyokuni, S., Okamoto, K., Yodoi, J. and Hiai, H., **1995**. Persistent oxidative stress in cancer. *FEBS letters*, 358(1), pp.1-3.

Vanitha, M.K., Sakthisekaran, D. and Anandakumar, P., Breast Cancer: Types, Epidemiology & Aetiology–A.

Villar, V.H., Merhi, F., Djavaheri-Mergny, M. and Durán, R.V., **2015**. Glutaminolysis and autophagy in cancer. *Autophagy*, 11(8), pp.1198-1208.

Wang, D., Feng, J.F., Zeng, P., Yang, Y.H., Luo, J. and Yang, Y.W., **2011**. Total oxidant/antioxidant status in sera of patients with thyroid cancers. Endocrine-related cancer, 18(6), pp.773-782.

Warburg, O. and Nguyen, T., **2015**. The Prime Cause of Cancer (Vol. 2). EnCognitive.com.

Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB, Thompson CB. **2008**. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc NatlAcad Sci U S A.* 105: 18782-18787.

Wiseman, M., **2008**. The second World Cancer Research Fund/American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. *Proceedings of the Nutrition Society*, 67(03), pp.253-256.

Wu, R., Feng, J., Yang, Y., Dai, C., Lu, A., Li, J., Liao, Y., Xiang, M., Huang, Q., Wang, D. and Du, X.B., **2017**. Significance of Serum Total Oxidant/Antioxidant Status in Patients with Colorectal Cancer. PloS one, 12(1), p.e0170003.

Yoshikawa T., Naito Y. **2002**. What Is Oxidative Stress? *JMAJ,* 45(7): 271–276.

Yücel A F., Kemik Ö., Kemik As., Purisa S. and Tüzün İ S. **2012**. Relationship between the Levels of Oxidative Stress in Mesenteric and Peripheral Serum and Clinicopathological Variables in Colorectal Cancer. *Balkan Med J,* 29**,** 144-7.

Zarrini, A.S., Moslemi, D., Parsian, H., Vessal, M., Mosapour, A. and Kelagari, Z.S., **2016**. The status of antioxidants, malondialdehyde and some trace elements in serum of patients with breast cancer. Caspian journal of internal medicine, 7(1), p.31.

Zmuda, J.M., Cauley, J.A., Ljung, B.M., Bauer, D.C., Cummings, S.R., Kuller, L.H. and Study of Osteoporotic Fractures Research Group, **2001**. Bone mass and breast cancer risk in older women: differences by stage at diagnosis. *Journal of the National Cancer Institute*, 93(12), pp.930-936.

Zowczak-Drabarczyk, M.M., Murawa, D., Kaczmarek, L., Połom, K. and Litwiniuk, M., **2013**. Total antioxidant status in plasma of breast cancer patients in relation to ERβ expression. Contemp Oncol (Pozn), 17(6), pp.499-503.

10.ANNEXES

10.1. Questionnaire

Dear respondents the aim of this questionnaire is to collect the necessary data to evaluate glutamate dehydrogenase, lactate dehydrogenase, ratios of reduced to oxidized glutathione and the oxidative stress index among breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia.

Therefore you are kindly requested to give correct information accordingly. Thank you for your time and keenness.

I. Personal information

1. Card no.__________________
2. Age (in yrs.) ______________
3. Gender

   Male ☐ Female ☐
4. Residential area

   ☐ Urban ☐ Rural

1.1. Education Level: Illiterate _____ High School or less_____ college or above______

II. Body Mass Index

1. Weight (in Kg) ________________
2. Height (m)_____________________
3. BMI___________________________

III. Physical Activity

1. Do you perform physical exercise?

   ☐Yes ☐No
2. If Yes, how often per week?

   ☐Occasionally ☐ 4days ☐ 3days ☐ 2days ☐other________

IV. Alcohol consumption

1. Do you consume alcohol?

   ☐ Yes ☐ No
2. If yes, how often?

   ☐Occasionally ☐1 Bottle alcohol daily ☐ 2-5 Bottle alcohol daily ☐other____

V. Smoking

1. Do you smoke?

   ☐Yes ☐No
2. If yes, how often?

   ☐Occasionally ☐1 cigar daily ☐ 1 pack per day ☐ other________

VI. Reproductive history

1. Age at menarche: ___________ Years

2. Menstrual status: Pre ☐ Post ☐

3. Age at menopause: __________ Years

4. Oral contraceptive use: Yes ☐ No ☐

4.1. About how old were you when you *first went on* the pill? _____Years

4.2. About how old were you when you *last came off* the pill? _____Years

VII. Personal and family history of breast cancer

1. Have you ever had certain breast cancer conditions before?

☐Yes ☐No

2. Have any of your close blood relatives ever had breast cancer?

☐Yes ☐No

3. If yes, whom

☐, one of your first degree relatives (mother, sister or daughter)

☐, two of your first degree relatives (mother, sister or daughter)

☐, any of your distantly related relatives (specify) -------------------------------------

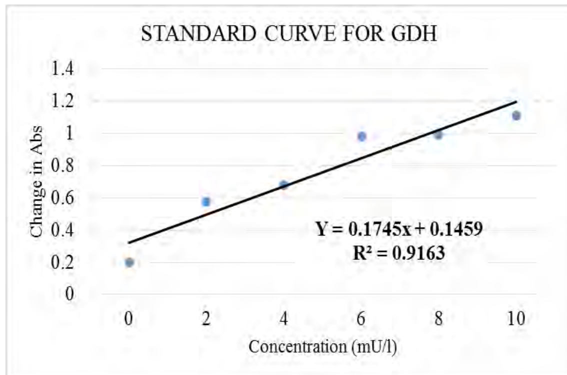
4. Have you ever had radiation therapy to the chest area as treatment before?

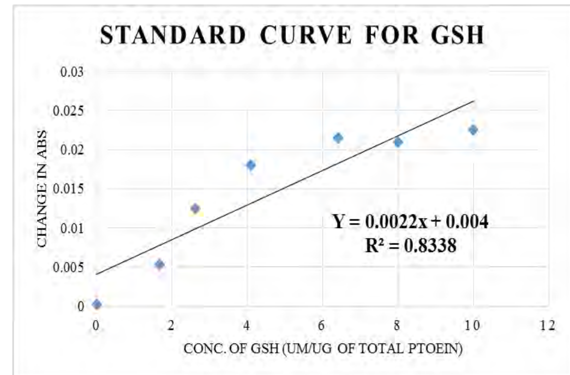
☐Yes ☐ No

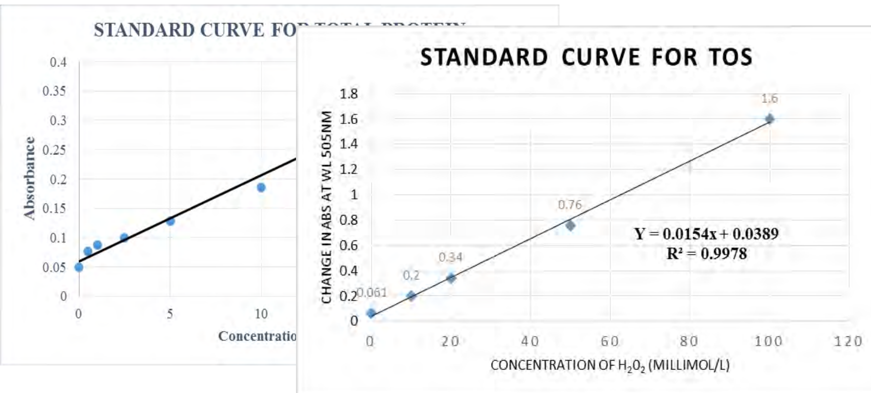
VIII. Pathological information (tumor characteristics)

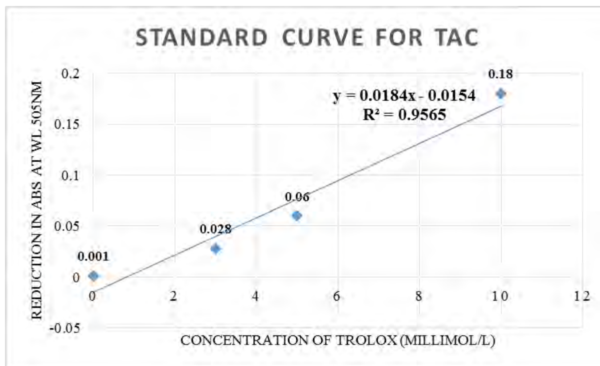
1. Tumor type (stage)________________________
2. Histological type (grade)___________________
3. Sites of breast cancer______________________

10.2. Standard Curve









10.3. Information sheet in English Version

Title of the Research Project: changes in glutamate dehydrogenase, lactate dehydrogenase, ratios of reduced to oxidized glutathione and the oxidative stress index among breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia.

Principal Investigator: Mohammed Mehdi (BSc, MSc candidate)
Name of the Organization: Addis Ababa University College of Health Sciences School of Medicine Department of Biochemistry.

Introduction

You are invited to participate as a study subject in a research conducted by MSc candidate, from Addis Ababa University. Your participation is voluntarily. The research teams will include one principal investigator, three advisors; two from Addis Ababa University biochemistry department and one from oncology unit. Please take as much time as you need to read or listen in the information sheet.

Purpose of the Research Project

We are asking you to take part in this study because we will try to compare glutamate metabolism, the oxidative stress level and free radical change among breast cancer patients so that we will suggest best strategy for treatment option of breast cancer.

Procedures and what will be expected from you for participation

In order to perform the indicated study at the five hospitals of Addis Ababa, Ethiopia, you are invited to take part in this project. If you are willing to participate, you need to understand the purpose of the study and give your consent. Not only this but also specimen collected from you will be used for the research purpose, and the results of your sample will be exposed to some concerned professional staffs as it is needed. The required clinical sample will be collected by a principal investigator and nurses of oncology department. Then, you are requested to give your consent to the sample collector. After consent, 3ml blood specimen will be collected from you by specimen collector and face to face interview for additional questions.

Potential risks and Discomforts

There will be minor discomfort during blood specimen collection. During collection of specimen from you, appropriate precaution will be taken and all samples will be collected by trained health professionals. If anything happened, appropriate medical care will be provided to you.

Confidentiality

We respect your privacy and confidentiality. Any information that identifies you will not be shared with anyone else outside the study team. The information we will collect from you as part of the study will be kept in a locked file cabinet, or be protected by a password on the computer only accessible to personnel involved in the study. There is no sensitive issue that you will be asked related with your social desirability but any information that is obtained in connection with this study and that can be identified with you will remain confidential.

Potential benefits to subjects and/or to the society

You will not receive any payment for your participation in this research study as compensation. But based on the diagnosis result you will be treated in view of that. In addition, the result of the study will be beneficial for the detection and managing of breast cancer. Hence, you are indirectly benefiting other patients and the society in this respect.

Participation and Withdrawal from the Study

The participation is completely voluntary and you have the right not to participate in this study. You may withdraw at any time and place without consequences of any kind. You may also reject to give any sample. You can ask any questions regarding to this study and you have a right to get a laboratory diagnosis result for free.

Contact information

If you have any questions about this study you can contact the following principal investigators and advisors for further information.

Moahmmed Mehdi **Phone:** 0910066838 **E-mail:** mohammed.mehdi@aau.edu.et

10.4. Information sheet in Amharic version

የተሳታፊዎች ፈቃድና መተማመኛ ቅፅ

በአዲስ አበባ ዪኒቨርሲቲ ጤና ሳይንስ ኮሌጅ የሕክምና ባዮኬሚስትሪ ት/ክፍል በማስተርስ ድግሪ ተማሪ የመመረቂያ ጥናት ላይ እዲሳተፉ ተጋብዘዋል፡፡እባክዎ በዚህ ጥናት ለመሳተፍ ከመስማማትዎ በፊት ከዚህ ቀጥሎ የሚገኘዉን ምንባብ በጥሞና ያንብቡና ግልጽ ያልሆነልዎትን ማንኛዉም ሃሳብ ይጠይቁ፡፡

መግቢያ

የጥናቱ ርዕስ changes in glutamate dehydrogenase, lactate dehydrogenase, ratios of reduced to oxidized glutathione and the oxidative stress index among breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia. የጥናቱ አላማ የጡት ካንሰር ህመምተኞችን ላይ በሽታውን ለመከታተል(የማዳን ሂደቱን ለማየት) የሚጠቅም ወይም የሚያስችል አዲስ ጥናት ነው፡፡
እናም እርስዎ በዚህ ጥናት ለመሳተፍ ጠቀሚና ምቹ ሆነው ተመርጠዋል፡፡ የእርስዎ በዚህ ጥናት ላይ የሚኖርዎት ተሳትፎ ሙሉ በሙሉ በበጎ ፈቃደኝነት ላይ የተመሰረተ ነዉ፡፡ በዚህ ጥናት ዉስጥ ላለመሳተፍ ወይም ለመሳተፍ ከወሰኑ በኀላ ለማቋረጥ የሚወስኑ ቢሆንም እንኩዋ በዚህ ሆስፒታል የሚሰጠዉ ማንኛዉም አገልግሎት አይቋረጥም፡፡በጥናቱ ለመሳተፍ የሚስማሙ ከሆነ የስምምነት ቅጹ ላይ በጹሁፍ ወይም በጣት ፊርማ ማስቀመጥ ይጠበቅዎታል፡፡

የጥናቱ ተሳታፊ ለመሆን የሚጠበቅበዎት ምንድን ነው?

በዚህ ጥናት ለመሳተፍ የሚስማሙ ከሆነ የደም ናሙና እንደሚወሰድና ለጥናቱ እንዲሚዉል መስማማት ይጠበቅብዎታል፡፡ ከተወሰደዉ ናሙና ላይ የሚገኙ መረጃዎች ከዚህ ሆስፒታል ዉጭ ለሚገኙና ለስራዉ አግባብነት ላላቸዉ ሰዎች ቢነገር የማይቃወሙ መሆኑን መስማማት ይጠበቅብዎታል፡፡ይሁን እንጅ ይህ አይነቱ መረጃ የርስዎን ማንነት የሚገልጡ መረጃዎችን ማለትም ስም፤አድራሻና የስልክ ቁጥር የመሳሰሉትን መረጃዎቸን አይጨምርም፡፡ ይልቁንም ለዚህ አገልግሎት ብቻ የሚዉል አርስዎን ለማወቅ የሚያስችል መለያ ቁጥር ጥቅም ላይ እንዲዉል ይደረጋል፡፡በተጨማሪም ስለርስዎ አጠቃላይ የጤና ሁኔታ ለሚቀርቡ አንዳንድ ተጨማሪ ጥያቄዎች መልስ መስጠት፡፡

በዚህ ጥናት መሳተፍ የሚያስከትላቸዉ ቸግሮች ምንድን ናቸዉ?

ናሙና በሚሰበሰብበት ወቅት ምንም አይነት የከፋ ችግር አያጋጥምዎትም፡፡ ነገር ግን ደም ሲወሰድ መጠነኛ የህመም ስሜት ሊያስከትል ይችላል፡፡ሆኖም ግን ናሙናዉን ለመሰብሰብ ልምድ ያለዉ ባለሙያ ስለሚመደብና አስፈላጊዉ የጥንቃቄ እርምጃ ስለሚወሰድ የህመም ስሜት አይኖርም፡፡

የህክምና መረጃ በሚስጥር ተጠብቆ መቆየት የሚችለዉ እንዴት ነዉ?

ስለራስዎ የሰጡት ማንኛዉም መረጃና ከተወሰደዉ ናሙና ላይ የተገኘዉ የላቦራቶሪ ዉጤት የሚዉለዉ ለጥናቱ አላማ ብቻ ነዉ፡፡ ይህን ማህደር ሊያገኙ የሚችሉት የተወሰኑ የጥናቱ ተባባሪ ሰዎች ብቻ ናቸው፡፡ከዚያም በላይ ስለ እርስዎ ያለውን ማንኛውንም መረጃ የተለየ የይለፍ ቃል ባለው የኮምፒውተር የመረጃ ማህደር ውስጥ እንዲቀመጥ ይደረጋል ፡፡

በዚህ ጥናት መሳተፍ የሚያስገኛቸው ጥቅሞች ምንድን ናቸው ?

ይህ ጥናት የማስተርስ ዲግሪ መመረቂያ እንደመሆኑ መጠን በዚህ ጥናት በመካፈልዎ በገንዘብ የሚያገኙት ጥቅም ባይኖርም ከጥናቱ በሚገኘው ውጤት ግን ተጠቃሚ ነዎት፡፡ የእርሶዎ ተሳትፎ የእርስዎንና የወገንዎትን የኩላሊት በሽታ ለማወቅና ለማከታተል ከፍተኛ ጥቅም ይኖረዋል፡፡

በዚህ ጥናት ተሳታፊ የመሆንዎ መብቶች ምንድን ናቸው ?

በዚህ ጥናት መሳተፍ ሙሉ በሙሉ በእርስዎ ፈቃደኝነት የተመሰረተ በመሆኑ በማንኛውም ሰዓትና ቦታ የማቋረጥ ሙሉ መብት የተጠበቀ ከመሆኑም በላይ እራስዎን ከጥናቱ በማግለልዎ ምክንያት የሚቀርብዎት ምንም አይነት የሆስፒታል አገልግሎት አይኖርም ፡፡ከዚህም በተጨማሪ ጥናቱን በተመለከተ ማንኛውንም አይነት ጥያቄ የመጠየቅና ገለጻ የማግኘት መብት አለብዎት፡ ፡የላብራቶሪ ምርመራ ውጤቱንም በነጻ ማግኘት ይችላሉ፡፡ ነገር ግን እርስዎ በሚሰጡን መረጃ የችግሩን ስፋት ለመከላከል እና ለመቆጣጠር ጠቃሚ ስለሆነ ለሚቀርብልዎት ጥያቄ ቀጥተኛ መልስ ይሰጡን ዘንድ በታላቅ አክብሮት እንጠይቃለን፡፡

ጥያቄ ካለኝ ወይም ችግር ቢያጋጥመኝ ምን ማድረግ ይገባል?

ይህንን ጥናት በተመለከተ ወይም ከዚህ ጥናት ጋር በተዛመደ መልኩ ስለሚያጋጥሙ ድንገተኛ አደጋዎች ወይም ጥያቄ ካለዎት በሚመለከተው አድራሻ ይጠቀሙ፡፡

መህመድ መህዲ

ሞባል፡ +251-910-066-838

ኢሜል፡ mohammed.mehdi@aau.edu.et

10.5. Informed consent form in English version

Code number…..…………………

I had been informed that the objective of this study is to evaluate changes in glutamate dehydrogenase, lactate dehydrogenase, and ratios of reduced to oxidized glutathione and the oxidative stress index among breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia. The results of this study have an importance to treat me and other patients, and to be used as an input for the future development of strategies or guidelines for follow up and treatment of breast cancer in Ethiopia. I had been also informed about the confidentiality of this study. The principal investigator requested me to participate in the study that would require my willingness to provide the required data that include blood sample and filling questionnaire. Therefore, with full understanding of the importance of the study, I agreed voluntarily to provide the requested samples and my benefit will be only from the free laboratory investigation result/s.

I_____________________________________ hereby give my consent for providing the requested information and specimens as the doctors find best for me.

Signature: ________________________________ Date_______________________

10.6. Informed consent form in Amharic version

የተሳታፊዎች ስምምነት ማረጋገጫ

የሚስጥር ቁጥር ----------------------------

የተሳታፊዉ ስም -----------------------------

እኔ ስሜ ከላይ የተጠቀሰው ተሳታፊ “changes in glutamate dehydrogenase, lactate dehydrogenase, ratios of reduced to oxidized glutathione and the oxidative stress index among breast cancer patients attending referral hospitals of Addis Ababa, Ethiopia” ጥናት ላይ በቂ ገለጻ ተደርጎልኛል፡፡ ለጥናቱም የደም ናሙና እንደሚያስፈልግ ተገልጾልኛል፡፡የጥናቱንም አላማዎችም ተረድቻለሁ፡፡
በመጠይቁ ላይ የገለጽኳቸው መረጃዎች በሙሉ በሚስጥር የተጠበቁ እንደሚሆኑ ተነግሮኛል፡፡ በጥናቱ ላይ ያለመሳተፍና ማንኛውንም መረጃ ያለመስጠት እንዲሁም በማንኛውም ጊዜ ከጥናቱ ራሴን የማግለል መብቴ የተጠበቀ እንደሆነ ተገልጾልኛል፡
፡

ስለዚህ ለዚህ ጥናት መረጃና የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍጹም ፍቃደኝነት ነው፡ በተጨማሪም ጥያቄ ለመጠየቅ ተፈቅዶልኝ ለማወቅ የፈለኩትን ያህል ማብራሪያ አግኝቻለሁ፡፡ የዚህ ጥናት ተሳታፊ በመሆኔ የማገኘው ጥቅም የሁሉንም ምርመራ ውጤት በነጻ ማግኘት እንደሆነ ተረድቻለሁ፡፡

በአጠቃላይ እኔ ከላይ በመተማመኛ ቅፅ የተጠቀሱትን ሁሉ በሚገባና በተረጋጋ መንፈስ አንብቤዋለሁኝ፡፡ ስለዚህ በዚህ ጥናት ለመሳተፍ ፈቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ፡፡

ፊርማ-------------------- ቀን ----/---/----------

(የስምምነት ቅጹን ማንበብ ለማይችሉ ተሳታፊዎች)

የአማካሪ ነርስ ስም -- ፊርማ -----------------

ቀን----------------------